

PROTOPLAST FUSION OF *CITRUS* FOR ROOTSTOCK
AND SCION IMPROVEMENT WITH EMPHASIS ON
WIDE HYBRIDIZATION

By

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Abstract of Dissertation Presented to the Graduate School
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Protoplast culture following polyethylene-glycol (PEG)-induced fusion resulted in the regeneration of intergeneric somatic hybrid plants from the following combinations: 'Succari' sweet orange [*Citrus sinensis* (L.) Osbeck] + *Severinia disticha* (Blanco) Swing.; 'Hamlin' sweet orange [*Citrus sinensis* (L.) Osbeck] + *S. disticha*; 'Valencia' sweet orange [*Citrus sinensis* (L.) Osbeck] + *S. disticha*; 'Nova' mandarin-tangelo [*Citrus reticulata* Blanco x (*Citrus reticulata* Macf. x *C. paradisi*)] + *S. disticha*; 'Succari' sweet orange + *Severinia buxifolia* (Poir.) Tenore; 'Succari' sweet orange + *Atalantia ceylanica* (Arn.) Oliv.; 'Succari' sweet orange + *Feronia limonia* (L.) Swing.; and 'Nova' mandarin-tangelo [*C. reticulata* x (*C. reticulata* x *C. paradisi*)] + *Citropsis gillettiana* Swing. & M. Kell.. The

somatic hybrid between 'Succari' sweet orange and *F. limonia* is the first report of successful hybridization of this wild species with *Citrus*, by any method.

Fusion experiments also resulted in the regeneration of the following interspecific somatic hybrids: 'Succari' sweet orange with 'Dancy' tangerine (*Citrus reticulata* Blanco), 'Minneola' tangelo (*Citrus paradisi* Macf. x *Citrus reticulata* Blanco), 'Murcott' tangor (purported *Citrus reticulata* X *Citrus sinensis*), 'Page' tangelo [*Citrus reticulata* Blanco x (*Citrus paradisi* Macf. x *C. reticulata*)], and 'Ponkan' mandarin (*Citrus reticulata* Blanco); 'Valencia' sweet orange with 'Minneola' tangelo, 'Murcott' tangor (purported *C. reticulata* X *C. sinensis*), and 'Page' tangelo; 'Hamlin' sweet orange with 'Ponkan' mandarin; and 'Rohde Red Valencia' sweet orange [*Citrus sinensis* (L.) Osbeck] with 'Dancy' tangerine.

Protoplasts of the first parental donor were isolated from either ovule-derived embryogenic calli or ovule-derived embryogenic suspension cultures, and protoplasts of the second parental donor were isolated from seedling leaves. Somatic hybrid plants were identified on the basis of leaf morphology, root tip chromosome number, and molecular marker analyses (isozyme or RAPD banding patterns).

All somatic hybrid plants produced in this research have been propagated in the greenhouse and will be evaluated in commercial field trials. Intergeneric combinations may show promise as *Citrus* rootstocks. Interspecific somatic hybrids,

besides having possible use as a scion variety, will also be used later as tetraploid breeding parents in interploid hybridization with selected monoembryonic diploid scions to produce improved seedless triploids with fresh market potential.

INTRODUCTION

World production of *Citrus* in 1993/94 was estimated to be 73.5 million tons. The United States was the world's second largest producer, with 12.2 million tons, after Brazil, with 15.4 tons (FAO, 1994).

Citrus producing regions roughly occupy a belt spreading approximately 35 °N and °S of the equator, but the main commercial areas are in the subtropical regions at latitudes more than 20 °N or °S of the equator (Soost and Cameron, 1975).

Citrus, as well as many related genera of the sub-family Aurantioideae, family Rutaceae, are native to Southeast Asia, with phylogenetic branches extending through central China to Japan, through the East Indies to New Guinea and Australia, and through the Indian subcontinent to tropical Africa (Scora, 1975; Soost and Cameron, 1975; Swingle and Reece, 1967).

According to Webber et al. (1967), the first member of the *Citrus* group to become known to European civilization was the citron (*Citrus medica* L.), followed by the sour orange (*Citrus aurantium* L.), lemon [*Citrus limon* (L.) Burm. f.], and sweet orange [*Citrus sinensis* (L.) Osbeck]. The introduction of *Citrus* to the rest of the world, including the Americas,

South Africa, and Australia was done by the European colonial expansion. All present-day, commercially significant, *Citrus* fruit species originated in the Old World, with the only exception being the grapefruit (*Citrus paradisi* Macf.), which presumably arose as a natural hybrid in Barbados following introduction of pummelo [*Citrus grandis* (L.) Osbeck] and sweet orange by European colonists (Bowman and Gmitter, 1990a, b; Scora et al., 1982).

Citrus fruits vary greatly in size, shape, fruit rind color, juice quality and other factors. Other horticultural tree characteristics, such as tree vigor and size or tolerance to biotic and abiotic factors, also vary tremendously among the *Citrus* commercial varieties, species, and related genera. Although there is a large amount of variability evident in the *Citrus* gene pool, available for *Citrus* breeding and cultivar improvement, most present-day cultivars widely grown in the main commercial producing areas of the world, originated as chance seedlings selections or bud sport mutations of existing cultivars (Soost and Cameron, 1975). The reason for this cannot be attributed to poor research or lack of good *Citrus* breeding programs, which have been carried out since 1893 by Swingle and Webber, in Florida (Cooper et al., 1962), for example. After the pioneering work of these two researchers, several other *Citrus* breeding and cultivar improvement programs have been developed, not only in Florida, but also in California and other countries besides the U.S.A., such as

Italy, Spain, South Africa, Brazil, France, Java, The Philippines, and Japan (Soost and Cameron, 1975). Some new varieties have been released during this century, following a great amount of work spent in the many conventional breeding programs. The main reason that more important new varieties have not been developed has been the many biological impediments, related to reproductive biology of *Citrus* and its relatives.

Most *Citrus* species and varieties exhibit asexual reproduction by seed, which is defined as apomixis. In the case of *Citrus*, apomixis is of nucellar origin and is classified as the facultative type. It is evident that the widespread occurrence of this kind of reproduction in much of this genus, which frequently results in polyembryonic seeds, has profound effects on *Citrus* breeding efforts (Cameron and Frost, 1968; Cameron et al., 1959; Parlevliet and Cameron, 1959; Soost and Cameron, 1975). These effects include obscuring taxonomic relationships, inheritance patterns, and sexual compatibilities; but the most obvious and critical effect of *Citrus* apomixis via nucellar embryony is that controlled hybridization using polyembryonic clones as maternal parents frequently yields few or no hybrid progeny either for selection or critically needed genetic studies (Soost and Cameron, 1975). Monoembryonic parents that produce only zygotic seedlings can be used to create segregating populations, but the number of such parents is small,

drastically limiting the pool of useful maternal parents (Grosser and Gmitter, 1990a).

High heterozygosity is another important genetic characteristic of *Citrus* in general, and much more information and understanding of the genetic control of important traits is needed (Soost and Cameron, 1975). As an important consequence, crosses made between complementary parents frequently fail to yield the desired recombinants in hybrid progeny. In other cases, weak or unhealthy progeny are produced as a result of strong inbreeding depression, which is frequently observed in such crosses (Barrett and Rhodes, 1976; Swingle and Reece, 1967; Torres, 1936).

Another important barrier to conventional *Citrus* breeding has been the absolute gametic (pollen and ovule) sterility, as well as self- and cross-incompatibility (Soost and Cameron, 1975). A system of gametophytic self- and cross-incompatibility has been observed in some *Citrus* species and interspecific hybrids (Soost, 1964, 1969; Ton and Krezdorn, 1966). Such incompatibilities have prevented several potentially useful hybridizations from being successful (Grosser and Gmitter, 1990a). Sexual hybridization between *Citrus* and related genera has been rare, with the exception of a few closely related genera, leaving several more distant relatives, possessing traits of potential value, that cannot be hybridized with *Citrus* (Barrett, 1977; Iwamasa et al., 1988; Swingle and Reece, 1967; Sykes, 1988).

A long period of juvenility is characteristic of *Citrus* seedlings and their immediate budded progeny. Most *Citrus* species have taken five years or more until first flowering and fruit production (Soost and Cameron, 1975). In addition to this fact, juvenility in *Citrus* is associated with thorniness, upright growth habit, and great vigor, resulting in large tree size. Because of juvenility and large tree size, hybrid evaluation is very costly (Grosser and Gmitter, 1990a); and, together, these factors decrease the rate of response to selection and increase the cost per unit of advancement (Hansche, 1983).

Many of the impediments or barriers to conventional *Citrus* hybridization have been overcome lately. The development of new biotechnologies combined with conventional strategies of plant breeding have resulted in the production of new *Citrus* hybrids with potential for cultivar improvement (Grosser and Gmitter, 1990a).

The primary objective of the present research was to produce somatic hybrids between *Citrus* and its relatives that possess potentially useful horticultural traits to be incorporated into the *Citrus* gene pool, using the technique of protoplast fusion.

Other objectives of this research work were

(a) Search for alternative sources of protoplasts of *Citrus* relatives to facilitate protoplast fusion experiments involving such species.

(b) Attempt to produce interspecific somatic hybrids between sweet orange and mandarin varieties to be used as tetraploid parents for scion improvement.

(c) Attempt to use and test recently developed new molecular marker techniques for somatic hybrid verification and identification, such as DNA amplification by polymerase chain reaction with random primers (Random Amplified Polymorphic DNA) (RAPD analysis).

REVIEW OF LITERATURE

Botany and Horticultural Characteristics of *Citrus* and its Relatives

Botanical Classification of the Genus *Citrus* and its Relatives

Citrus species and their close and remote relatives belong to the family Rutaceae, subfamily Aurantioideae (Swingle and Reece, 1967). Table 1 shows the tribes, subtribes and genera in the subfamily Aurantioideae as classified by Swingle and Reece (1967). The subfamily is divided in two tribes (I, Clauseneae, very remote and remote citroid fruit trees, and II, Citreae, citrus and citroid fruit trees), each with three subtribes. The genus *Citrus*, with 16 species is classified in the subtribe 2, Citrinae, of the tribe 2, Citreae.

Direct Use of *Citrus* Relatives as Rootstocks and the Potential of Selected Genera to be Exploited in Rootstock Improvement

As can be observed in Table 1, there is a great genetic diversity in the *Citrus* subfamily, which can potentially be used in *Citrus* improvement programs.

Some research work has reported the main characteristics of the many genera and species of the subfamily Aurantioideae and emphasized several qualities of this germplasm. One of the

Table 1: List of tribes, subtribes, and genera of the subfamily Aurantioideae (Swingle and Reece, 1967).

Tribes	Sub-tribes	Genera	
I. Clauseneae	Micromelinae	Micromelum	(9 spp.)
	Clauseninae	Glycosmis	(35 spp.)
		Clausena	(23 spp.)
		Murraya	(11 spp.)
	Merrilliinae	Merrillia	
II. Citreae	Triphasiinae		
	(A. Wenzelia Group)		
		Wenzelia	(9 spp.)
		Monanthocitrus	
		Oxanthera	(4 spp.)
		Merope	
	(B. Triphasia Group)		
		Triphasia	(3 spp.)
		Pamburus	
	(C. Luvunga Group)		
		Luvunga	(12 spp.)
		Paramignya	(15 spp.)
	Citrinae		
	(A. Primitive Citrus Fruit Trees)		
		Severinia	(6 spp.)
		Pleiospermium	(5 spp.)
		Burkillanthus	
		Limnocitrus	
		Hesperethusa	
	(B. Near-Citrus Fruit Trees)		
		Citropsis	(11 spp.)
		Atalantia	(11 spp.)
	(C. True Citrus Fruit Trees)		
		Fortunella	(4 spp.)
		Eremocitrus	
		Poncirus	-
		Clymenia	
		Microcitrus	(6 spp.)
		Citrus	(16 spp.)
	Balsamocitrinae		
	(A. Tabog Group)		
		Swinglea	
	(B. Bael-Fruit Group)		
		Aegle	
		Afraegle	(4 spp.)
		Aeglopsis	(2 spp.)
		Balsamocitrus	
	(C. Wood-Apple Group)		
		Feronia	
		Feroniella	(3 spp.)

first studies done in this respect was carried out by Bailey (1922), in which several species of the genus *Citrus* and other related genera are described, including *Poncirus*, *Microcitrus*, *Eremocitrus*, *Hesperethusa*, *Citropsis*, *Muraya*, *Pamburus*, *Clausena*, *Aegle*, and *Pleiospermium*.

The direct use of *Citrus* relatives as rootstocks has been attempted several times. Cooper et al. (1957) did some of the pioneering work in this area, in which the viability of the use of other genera and *Citrus* hybrids as rootstocks was investigated. The results showed good performance of *Severinia buxifolia* (Poir.) Tenore as a rootstock for grapefruit. In comparative studies, this species did not show any symptoms of iron deficiency or boron toxicity, and showed good adaptation in saline soils. A latter work performed by Cooper (1961) also confirmed the excellent adaptability of *S. buxifolia* to saline soils.

Adaptation to saline soils is also found among other related genera, besides *Severinia*, such as *Merope* (Triphasiinae) (Bitters et al., 1969). Goell (1969), in Israel, demonstrated that *Eremocitrus glauca* (Lindl.) Swing. was resistant to high salt concentrations and accumulated low amounts of chloride ions in leaves. Research carried out in India also showed salt tolerance of Karna Khatta (*Citrus karna* Raf.) (Bhambota and Kanwar, 1969).

Wutscher et al. (1970) described the results of an experiment involving 16 rootstocks (including 8 hybrids) for

grapefruit (*C. paradisi*), in alkaline soils, in Texas. Trees on *S. buxifolia* rootstock showed vegetative growth less than desirable when compared with other rootstocks. These plants also showed chlorosis symptoms which were suggested to be due to high CaCO_3 concentration in the soil and high magnesium/iron (Mg/Fe) ratio as detected by foliar analysis. On the other hand, trees budded on *S. buxifolia* showed extremely low foliar boron (B) content. In another paper, Wutscher and Shull (1976) also describe the performance of eleven rootstocks using 'Marrs' early orange [*Citrus sinensis* (L.) Osbeck] as a scion, emphasizing the positive influence of *S. buxifolia* on tree vigor, yield, and leaf nutrient concentration.

Feder and Ford (1957) carried out an investigation in Florida on the seedling tolerance of several *Citrus* species and related genera to the burrowing nematode *Radopholus similis* (Cobb) Thorne. *Citrus ichangensis* Swing. and *Microcitrus australis* (Planch.) Swing. were resistant to this nematode under the conditions of the experiment. Cleopatra mandarin (*Citrus reticulata* Blanco) and Morton citrange [*Poncirus trifoliata* (L.) Raf. x *Citrus sinensis* (L.) Osbeck] were considered tolerant whereas rough lemon (*Citrus jambhiri* Lush.) and 'Minneola' tangelo (*Citrus paradisi* Macf. x *Citrus reticulata* Blanco) were found to be susceptible to the burrowing nematode. Follow up research work done by the same authors and co-workers showed good performance of other *Citrus*

rootstocks as well as the *Citrus* relative *Citropsis gillettiana* Swing. & M. Kell., considered resistant to the burrowing nematode (Ford and Feder, 1960, 1961; Ford et al., 1959).

Baines et al. (1960) investigated the susceptibility of some species and varieties of *Citrus* and some other Rutaceous plants to the citrus nematode (*Tylenchulus semipenetrans* Cobb.). The authors found out that the species *Aeglopsis chevalieri* Swing., *Afraegle paniculata* (Schum.) Engl., *Balsamocitrus dawei* Stapf., *Clausena lansium* (Lour.) Skeels, *Murraya paniculata* (L.) Jack, and *S. buxifolia* were free of the citrus nematode; *Atalantia citroides* Pierre ex Guill. was infected slightly, and *Cneoridium dumosum* very slightly. *Fortunella crassifolia* Swing. was infected moderately and *Microcitrus australasica* (F. Muell.) Swing. was severely infected by *T. semipenetrans*.

Other studies were developed in an attempt to evaluate the tolerance of many *Citrus* selections and related genera to the citrus nematode (*T. semipenetrans*) and the burrowing nematode (*R. similis*) (Hutchison and O'Bannon, 1972; O'Bannon and Ford, 1977; O'Bannon et al., 1977). Milam lemon (*Citrus jambhiri* Lush.), 'Pineapple' sweet orange [*Citrus sinensis* (L.) Osbeck], *M. australis*, and *M. australasica* were found to be resistant to the burrowing nematode. *Poncirus trifoliata* (L.) Raf., Swingle citrumelo [*Citrus paradisi* Macf. x *Poncirus trifoliata* (L.) Raf.], and *S. buxifolia* were some of the

examples that were considered resistant to the citrus nematode in these studies.

Hutchison et al. (1972) reported the results of an experiment in Florida in which 80 rootstock varieties were evaluated for their susceptibility to *Phytophthora parasitica* Dastur, *R. similis*, and *T. semipenetrans*. All *P. trifoliata* selections and one trifoliolate orange hybrid exhibited resistance to *P. parasitica* and *T. semipenetrans*. Carrizo, Rusk, and Troyer citranges [*Citrus sinensis* (L.) Osbeck x *Poncirus trifoliata* (L.) Raf.], three rough lemons (*C. jambhiri*), six sweet limes (*Citrus aurantifolia* (Christm.) Swing.), four Rangpur limes (*Citrus limonia* Osbeck), two Cleopatra mandarins (*C. reticulata*) and two 'Helseth' lemons were rated moderately resistant to *P. parasitica*. Under the conditions of the study, all entries were found susceptible to *R. similis*.

Venning (1957), in Cuba, reported the preliminary results of an experiment involving *Swinglea glutinosa* (Blanco) Merr. as a rootstock for *Citrus*. The *Citrus* relative was budded with several sweet orange [*Citrus sinensis* (L.) Osbeck], lemon (*C. limon*), acid lime [*Citrus aurantifolia* (Christm.) Swing.], and grapefruit (*C. paradisi*) varieties. Subsequent growth of the orange, grapefruit, and mandarin scions was much slower than their development on sour orange (*Citrus aurantium* L.). However, growth of all the acid *Citrus* varieties was much more vigorous than is obtained on any other stock. No graft

incompatibility was reported and the author also noted high tolerance of *S. glutinosa* to gummosis diseases (*Phytophthora* spp.).

Carpenter and Furr (1962), in California, carried out an experiment to evaluate the tolerance to root rot caused by *P. parasitica* in seedlings of *Citrus* and related genera. Approximately 30,000 young seedlings of 515 varieties and selections of *Citrus* and related genera were inoculated by immersing the roots in aerated water containing the fungus species. Tolerant seedlings were found in 31 species or groups of hybrids of the following genera: *Citrus*, *Eremocitrus*, *Fortunella*, *Microcitrus*, *Poncirus*, and *Severinia*. Among the *Citrus* species, Alemow (*Citrus macrophylla* Wester), rough lemon (*C. jambhiri*), Volkamer lemon (*Citrus volkameriana* Ten. and Pasq.), and pummelo (*C. grandis*) showed the greatest tolerance to *P. parasitica*.

Other authors have also investigated the resistance of several *Citrus* rootstocks, including some related genera, to *P. parasitica* (Grimm and Hutchison, 1973, 1977; Hutchison and Grimm, 1972, 1973). Among these studies, *C. macrophylla*, *Citrus miaray*, *S. buxifolia*, 'Cuban' shaddock, 'Ogami' pummelo (*C. grandis*), sweet lime (*C. aurantifolia*), trifoliate orange (*P. trifoliata*) and its hybrids, and a sour orange hybrid were found to be resistant.

In another study, Broadbent (1969) found rough lemon and Karna khatta (*C. karna*) to be susceptible, and *P. trifoliata*,

S. buxifolia, and *M. australasica* to be resistant to zoospore attraction of *Phytophthora citrophthora*.

Some research has also been done in the area of *Citrus* cold hardiness. Several studies have been carried out in an attempt to evaluate different *Citrus* selections and some related genera as cold tolerant rootstocks for *Citrus*. Among these studies the following can be cited: Cooper (1952); Gardner and Horanic (1958); Witt et al. (1988); Yelenosky (1976); Yelenosky and Hearn (1967); Yelenosky and Young (1977); Young (1977); Young and Olson (1963) and the review by Yelenosky (1985).

Bitters et al. (1964, 1969) described the main characteristics of most genera of the *Citrus* subfamily. The authors indicated the potential of some genera such as *Hesperethusa*, *Eremocitrus*, *Microcitrus*, *Citropsis*, *Clausena*, *Clymenia*, *Atalantia*, and *Swinglea* as valuable sources of germplasm to be utilized in *Citrus* breeding programs. In another study, Bitters et al. (1973) describe in detail the species and their hybrids of the Papeda group, emphasizing their use as *Citrus* rootstocks. The authors suggested more detailed investigation of the species *C. macrophylla*, *C. ichangensis*, *C. webberii* var. *montana*, and 'Yuzu' orange (*Citrus ichangensis* Swing. x *Citrus reticulata* Blanco).

The genus *Atalantia* has also been evaluated in Florida. Campbell (1979) emphasized the excellent overall pest and

disease resistance of *Atalantia monophylla* DC., besides its good adaptation to calcareous soils of southern Florida.

Hearn et al. (1974) discussed the several factors to be considered in a *Citrus* rootstock breeding program, emphasizing as main pest problems, nematodes, CTV, and blight, and as physiological factors, adaptations to saline soils, yield and fruit quality, tolerance to low temperature and tree size control. The authors also describe the qualities and limitations of the main rootstocks commercially used in Florida and point to some *Citrus* relatives as sources of tolerance or adaptation to one or more of the factors discussed.

Other research has focused on development of rootstocks that can reduce the vigor and size of the *Citrus* tree, in order to reduce harvest costs and obtain better efficiency of the general grove maintenance jobs such as fertilization and spraying. Bitters et al. (1977) suggested some *Citrus* relatives that might accomplish reduction in tree vigor and they include the genera *Clymenia*, *Microcitrus*, *Eremocitrus*, and *Citropsis*.

Valle et al. (1981), in Cuba, report the results of rootstock experiments for 'Valencia' orange (*C. sinensis*). The authors discussed the influence of *S. buxifolia* on the reduction of tree size, and severe symptoms of graft incompatibility were observed. The same problem was also noted for *S. glutinosa* and *C. gillettiana* when these species were

used as rootstocks for sweet orange. However, the authors suggested further investigation of these species with the use of interstocks, in an attempt to exploit their qualities in reducing tree size.

In some good reviews, the importance of the *Citrus* relatives have been emphasized as an alternative source of germplasm for rootstock development. Among these works are the reviews of Castle (1987); Castle et al. (1993); Sykes (1988); Wutscher (1979). Other works have tried to emphasize characteristics of specific rootstocks commercially in use in the citrus industry, such as some selections of *P. trifoliata*, citrumelos (*C. paradisi* x *P. trifoliata*), and the Rangpur lime (*C. limonia*) (Castle et al., 1988; Cohen, 1970; Hutchison, 1974; Shannon et al., 1960).

Development of New Biotechnologies for Citrus Cultivar Improvement

General Considerations

As previously discussed, the recent development of new biotechnologies such as recovery of new plants through somatic embryogenesis from protoplast fusion experiments has created new horizons for *Citrus* cultivar development. The combination of these new techniques with conventional breeding strategies provides opportunities to overcome the traditional natural barriers to *Citrus* breeding encountered in its own reproductive biology.

Somatic hybridization using protoplast fusion produces tetraploid plants. In the case of *Citrus*, somatic hybrid plants will have genetic constitution of $2n=4x=36$ chromosomes, 18 chromosomes from each of the two diploid parents involved in the fusion. One direct consequence is that the somatic hybrid should not express deleterious nor beneficial recessive genes, that can be unmasked by meiotic segregation in conventional hybridization. Further, traits that are conditioned by dominant alleles in one of the donors should be expressed in the somatic hybrid, although this hypothesis awaits confirmation from the *Citrus* somatic hybrids (Grosser and Gmitter, 1990a). Other advantages of protoplast fusion include the possibility of production of somatic hybrids between sexually incompatible parents, and also to overcome the problem of apomixis and nucellar polyembryony (Grosser and Gmitter, 1990a).

Tissue Culture of *Citrus*

The citron (*C. medica*) was probably the first-*Citrus* species to be cultivated in vitro, with callus induced from stem sections when cultured in Gautheret's (1942) medium supplemented with 1-naphthaleacetic acid (NAA) (Démétriadès, 1954). Soon after, callus initiation from citron stem cultures was also reported using the nutrient medium of Heller (1953), supplemented with NAA and coconut milk (Bové and Morel, 1957). In both reports, subcultures of the citron callus took, on average, six weeks to double in fresh weight, with only 50-60%

colony survival. In another investigation, explants composed of the fruit mesocarp of citron showed the best callus induction on a nutrient agar containing indole-3-acetic acid (IAA) and gibberellic acid (GA_3) (Schroeder and Spector, 1957). The vigorous growth in vitro by callus from juice vesicles of lemon fruits was demonstrated by Kordan (1959), who used a liquid medium containing only sugar and minerals. Growth was most vigorous in the pH range 7.0 to 7.7 and occurred to a lesser extent at pH 6.0. Subcultures carried on mitotic activity for periods of up to 10 months and gained in fresh weight as much as 2600 percent.

Although *Citrus* tissue culture has been practised since the early 1950s, a good protocol for general culture was not available until 1969 with the basal medium developed by Murashige and Tucker (1969). The medium was a modification of the Murashige and Skoog medium (Murashige and Skoog, 1962), commonly used in tissue culture, and differed in these aspects: 3×10^{-6} M 2,4-D or 3×10^{-5} M NAA replaced the IAA (2 mg/l); kinetin was specified as 1×10^{-6} M; the B-vitamins thiamin-HCl; nicotinic acid, and pyridoxin-HCl were increased to 10, 5 and 10 mg/l, respectively; and sucrose was raised from 3% to 5%. The tissues were grown at 30 °C with continuous darkness. This medium enabled vigorous growth of callus derived from lemon juice vesicles (*C. limon*), with a 30-fold enlargement in a 4-week passage period. Rapid callus initiation was also achieved in freshly isolated grapefruit

(*C. paradisi*), citron (*C. medica*), shaddock (*Citrus grandis*), and sweet orange (*C. sinensis*) juice vesicles. However, the recultured callus of these showed markedly less growth than those of the lemon fruit. Orange juice was required as a supplement for vigorous growth of recultures.

Polyembryony is a biological phenomenon exhibited by many *Citrus* species and also has raised interest in tissue culture studies. Ranga-Swamy (1961) and Sabharwal (1962) were the first to recover adventive embryos in vitro from excised nucelli of polyembryonic *Citrus*. Later on, Rangan et al. (1968) reported the first successful induction of nucellar embryogenesis in vitro from monoembryonic *Citrus*. One year later, Rangan et al. (1969) successfully isolated and identified zygotic and nucellar embryos in vitro of *C. aurantium* seed (control pollinated with *P. trifoliata*, as a marker). Early heart-shaped embryos isolated from seeds 100-120 days post-pollination, were successfully cultured on a modified White's medium, supplemented with 400 mg/l casein hydrolysate. The developing embryos were germinated into seedlings on medium lacking sugar and the casein hydrolysate. Adventive embryos were also successfully obtained with tissue culture of the nucellus of Shaddock (*C. grandis*), 'Ponderosa' lemon (*C. limon*), and 'Temple' orange (*C. reticulata* x *C. sinensis*) in a modified Murashige and Skoog medium. Both zygotic and nucellar embryogenesis in vitro was achieved under

a constant temperature of 26.7 °C and daily 16-hour illumination with low intensity Gro-lux light.

All the studies of embryo induction from nucellar and ovular tissue were from direct embryo formation. In the early 1970s, Mitra and Chaturvedi (1972) described the first example of embryo formation from callus proliferated from the nucellus of *C. aurantifolia*. Another classic experiment in *Citrus* tissue culture was done in the early 1970s by Kochba et al. (1972), who attempted to determine the best culture medium, and nucellus and ovule age for tissue culture of three *Citrus* cultivars: 'Shamouti' and 'Valencia' oranges (*C. sinensis*) and 'Marsh Seedless' grapefruit (*C. paradisi*). Ovules from four-week-old fruits gave the best results in 'Shamouti' orange, while 'Valencia' orange and 'Marsh Seedless' grapefruit ovules from six- to eight-week-old fruits, and nucellus from eight-week-old fruit, were found to be the optimum for embryo development. Somatic embryos were formed on Murashige and Tucker nutrient medium, supplemented with 500 mg/l malt extract. Germinated embryos developed on the same basal medium supplemented with kinetin and indole-3-acetic acid (IAA), coconut milk or gibberellic acid (GA_3). The ratio of kinetin/IAA, and the addition of coconut water and GA_3 were also important in stem elongation and root formation. The authors also reported the production of embryogenic callus from ovular tissue of 'Shamouti' orange. A few years later, Button et al. (1974) performed structural studies in the same

kind of callus from 'Shamouti' orange. The results revealed that the callus differed from most plant calli, which normally are made of unorganized parenchymatous tissue. The 'Shamouti' orange callus was found to be made of small, compact, spherical nodules (between 0.1 and 1.0 mm in diameter) that were considered to be proembryos; the proembryos were able to develop into cotyledon-stage embryos, and eventually to form plantlets.

Grinblat (1972) reported the first successful attempt to establish growth of *C. madurensis* in vitro. Stem explants were obtained from six week-old seedlings grown in vitro, under aseptic conditions. Basal Murashige and Skoog medium supplemented with 100 mg/l benzyl adenine (BA), 0.1 mg/l naphthaleneacetic acid (NAA), and 500 mg/l malt extract (ME) induced the greatest initiation of buds. Roots were initiated in explants grown on basal medium supplemented with 0.1 mg/l NAA and 500 mg/l ME, and in medium lacking growth regulators. Plants were recovered from the explants.

Chaturvedi and Mitra (1974) were also successful in the micropropagation of *Citrus* through isolated stem and leaf callus of *C. grandis* and *C. sinensis*. The greatest number of shoots was produced with basal Murashige and Skoog medium supplemented with 0.25 mg/l 6 benzylamino purine (BA) + 0.1 mg/l naphthaleneacetic acid (NAA). Calli regenerated from excised shoots produced shoot-buds from their cut ends in this treatment and roots in a treatment containing 0.1 or 0.5 mg/l

NAA. Numerous rooted shoots developed into vigorous plants in potted soil.

After the first report of production of *Citrus* embryogenic callus from nucellar tissue, several other investigations were conducted in this area. Kobayashi et al. (1984) reported the induction of nucellar callus from eleven *Citrus* cultivars (eight sweet orange varieties - *C. sinensis*), *C. junos*, *C. deliciosa*, and 'Nova' mandarin-tangelo [*C. reticulata* x (*C. reticulata* x *C. paradisi*)]. The best medium for callus induction was the Murashige and Tucker (MT) medium supplemented with 10 mg/l 6-benzylaminopurine (BA). Calli induced from 'Trovita' orange ovule were maintained on MT medium with and without BA for five years by subculturing at four- to eight-week intervals. Green embryos, which eventually developed into plantlets, were induced from callus cultivated in MT medium without BA. Galactose, lactose, and coconut water were also found to stimulate embryogenesis.

Ollitrault et al. (1992) discussed the induction of embryogenic *Citrus* calli through ovule culture. The production of embryos was successfully achieved in all species studied: *C. aurantium*, *C. sinensis*, *C. reticulata*, *C. limon*, *C. aurantifolia*, *C. paradisi*, and *P. trifoliata*. Best results were obtained with ovules excised three weeks after anthesis and cultured in Murashige and Tucker (MT) medium without any plant growth regulator or in MT + kinetin. However, the

production of an embryogenic callus could not be achieved in the species *C. limon*, *C. aurantifolia*, and *P. trifoliata*.

Kochba and Button (1974) showed that callus age was another crucial factor for embryo stimulation. The authors considered three-week-old callus as a standard and found that six-week-old callus had ten times the capacity for embryo development, while fourteen-week-old callus had one hundred times the capacity for embryo induction. However, callus aged for twenty weeks lost the capacity for embryo development.

Habituation of the callus, i.e., callus not requiring exogenous auxins and cytokinins for growth, has been observed in some lines (especially 'Shamouti' sweet orange) after continuous subculture for several years (Kochba and Button, 1974; Kochba and Spiegel-Roy, 1977). Embryogenesis in such lines could be inhibited by IAA and NAA. Callus growth is usually weaker in the presence of IAA, while concentrations below 0.01 mg/l NAA stimulate callus growth. Embryogenesis can also be stimulated by auxin synthesis inhibitors such as 5-hydroxynitrobenzylbromide (HNB) and 7-aza indole (AZI). Cytokinins such as benzyladenine (BA), kinetin, and N⁶ [isopentyl] adenine (2iP) have been shown to depress embryogenesis (Kochba and Spiegel-Roy, 1977).

Kochba et al. (1978a) also demonstrated that abscisic acid (ABA) has a stimulatory effect on embryogenesis. In the same way, ethephon at concentrations as low as 0.1 mg/l has induced embryogenesis, while higher concentrations have

inhibited it. In addition, 2-chloroethyl trimethyl-ammonium chloride (CCC) and Succinic acid 2,2-methyl hydrazide (Alar) also stimulated embryogenesis. GA_3 has shown to suppress embryogenesis (Kochba et al., 1978a).

Embryogenesis in *Citrus* tissue culture has also been shown to be influenced by carbon source. Kochba et al. (1978b) reported that the galactose-yielding sugars raffinose and lactose can stimulate embryogenesis from lines known to be embryogenic as well as in lines from which no embryogenesis had been observed. In a later investigation, Kochba et al. (1982) discovered that the effect of the carbon source on embryogenesis differs among cultivars. 'Shamouti' sweet orange (*C. sinensis*) and 'Villafranca' lemon (*C. limon*) required 32 mM galactose and lactose for maximum induction, while 64 mM was optimum for 'King' orange (*C. nobilis*), and navel sweet orange (*C. sinensis*). Embryogenesis in sour orange (*C. aurantium*) was only stimulated by lactose. Sucrose enhanced embryogenesis only at low levels. For most cultivars the optimum concentration was less than 16 mM. Glucose and fructose were not very effective in stimulating embryogenesis.

Another carbon source, glycerol, also has been shown to stimulate embryo formation, even in cultures not yielding many embryos, but embryos had to be removed from the medium containing glycerol as soon as they appeared or they would turn brown and die (Ben-Hayyim and Neumann, 1983). Research performed by Gavish et al. (1991) also demonstrated highly

efficient synchronous embryogenesis in suspension cultures of sour orange (*C. aurantium*) induced by a change in the carbon source of the growth medium from sucrose to glycerol. A three-week period was required for embryo development. In contrast to the results achieved by Ben-Hayyim and Neumann (1983), Gavish et al. reported that embryo development showed an absolute requirement for the continued presence of glycerol.

Stimulatory effects of lactose and glycerol on growth and somatic embryogenesis of nucellar-derived callus of 'Meyer' lemon (*Citrus meyeri* Y. Tan.) have also been investigated (Singh, A.K. et al., 1992). Lactose (5%) and glycerol (2%) separately induced callus growth. Glycerol (1-4%) induced embryogenesis either poorly or not at all. Lactose (4-5%) induced embryo formation.

Moore (1985) examined the effects of the cultivar and growth additives on the induction of embryogenesis from undeveloped ovules from mature fruit of *Citrus*. Fifteen of 17 representative polyembryonic cultivars investigated produced embryos, but cultured ovules of five monoembryonic cultivars did not become embryogenic. One cultivar, 'Marsh' grapefruit (*C. paradisi*), was chosen to test the effects of growth additives on the induction of somatic embryogenesis from undeveloped ovules. A low concentration (0.01 mg/l) of butanedioic acid mono (2,2-dimethylhydrazine) (daminozide) was most effective at increasing the induction of somatic embryogenesis over the control treatment of 500 mg/l malt

extract. The presence of abscisic acid or malt extract (>100 mg/l in the medium) also increased embryo induction, but germination of embryos obtained on the malt extract medium was poor.

Gmitter and Ling (1991) and Gmitter et al. (1991) developed methods to produce nonchimeric autotetraploid *Citrus* plants via in vitro somatic embryogenesis in the presence of colchicine. Undeveloped ovules from immature fruit of 'Valencia' sweet orange (*C. sinensis*) and 'Orlando' and 'Minneola' tangelos (*C. reticulata* x *C. paradisi*) were cultivated on Murashige and Tucker medium supplemented with 500 mg/l malt extract and 0, 0.01%, or 0.1% colchicine for three weeks (Gmitter and Ling, 1991). The high colchicine concentration suppressed embryogenesis in tangelos. Colchicine treatments had no subsequent effect on embryo germination. Both diploid and tetraploid 'Valencia' and 'Orlando' plants were recovered, as revealed by chromosome root-tip number determinations. 'Minneola' cultures produced only diploid plants. Gmitter et al. (1991) also produced autotetraploid plants from colchicine-treated embryogenic calli of sweet orange. All tetraploid plants were nonchimeric.

Sauton et al. (1982) demonstrated the ability of 'Trovita' orange (*C. sinensis*), 'Eureka' lemon (*C. limon*), *P. trifoliata*, and Troyer citrange (*C. sinensis* x *P. trifoliata*) to regenerate shoots and form new plantlets from root meristem cultures. Regeneration occurred directly, without callus

formation, or indirectly, after callus formation. The presence of BAP (1 mg/l) and 2,4-D (0.1 mg/l) in the Murashige and Skoog medium was important for adequate shoot regeneration.

Benzyladenine at 10 μ M was also shown to be very important for shoot production in vitro from juvenile and mature nodes of Carrizo citrange (*C. sinensis* x *P. trifoliata*), trifoliolate orange (*P. trifoliata*), Cleopatra mandarin (*C. reticulata*), Rangpur lime (*C. limonia*), and 'Symon's' sweet orange (*C. sinensis*) (Barlass and Skene, 1982). Stem internodes also responded to this plant growth regulator, producing multiple shoots. Naphthaleneacetic acid at 10 μ M was found to be necessary for rooting in vitro-grown shoots.

Nito and Iwamasa (1990) reported plant regeneration from juice vesicle callus of satsuma mandarin (*C. unshiu*). Excised juice vesicles were placed in Murashige and Skoog medium supplemented with α -naphthaleneacetic acid (NAA), kinetin (K), and gibberellic acid for callus induction. Adventitious embryos arose from the callus tissue on the medium containing 1 mg/l NAA. Embryo germination and plant formation were observed in medium containing 1 mg/l GA.

Grosser and Chandler (1986) demonstrated that Swingle citrumelo (*C. paradisi* x *P. trifoliata*) could also be micropropagated in vitro from seedling nodal sections of 0.5 and 1.0 cm, and internodal segments. Recovery of whole plants of 0.5 cm required coumarin, which induced both root and

shoot formation. An optimum concentration of coumarin (90-150 μ M) increased the number of whole plants recovered per seedling approximately fivefold.

Recently, Grosser et al. (1993) developed an alternative way to propagate Cohen citrange, a hybrid produced at the University of Florida that bears seedless fruits. Due to the adaptability of this tree under challenging conditions, there has been interest in developing an alternative method of propagation for evaluation. Undeveloped ovules were removed from mature fruit and cultured in vitro, with Murashige and Tucker (MT) modified medium. Somatic embryo proliferation was observed. Embryos were transferred to different media for enlargement and germination. Shoot proliferation occurred in an MT medium supplemented with 3 mg/l BA, 0.02 mg/l 2,4-D, and 25 g/l sucrose. Shoots were rooted in 1/2 strength MT basal medium plus 0.02 mg/l NAA, 0.5 g/l activated charcoal, and 25 g/l sucrose.

Shoot tips (2 to 3 mm) from mature plants of 'Khasi' mandarin (*C. reticulata*) and 'Assam' lemon (*C. limon*) could also be propagated in vitro, when cultured on Murashige and Skoog (MS) medium, supplemented with 1.0 mg/l BAP, 0.5 mg/l kinetin and 0.5 mg/l NAA. Root induction was observed when seven-week-old single shoots (approximately 2 cm long) of both *Citrus* species were cultured on MS medium supplemented with 0.25 mg/l BAP, 0.5 mg/l NAA, and 0.5 mg/l IBA (Singh, S. et al., 1994).

Hossain et al. (1993) developed a protocol for plant regeneration through organogenesis of nucellar explants excised from fertilized ovules of immature fruits of *Aegle marmelos* (L.). Adventitious shoots were initiated on Murashige and Skoog (MS) medium containing various combinations of BA, NAA, IAA, and GA. Medium containing 4.4 μM BA and 2.7 μM NAA produced the maximum number of adventitious buds per explant. A low concentration of BA (0.44 μM) was required for shoot elongation. Regenerated shoots were rooted in half-strength MS medium with 4.9 μM IBA.

Plant recovery from anther culture has also been reported in *Citrus*. Chaturvedi and Sharma (1985a, b) described the production of androgenic plants of *C. aurantifolia*, when uninucleate pollen grains at the tetrad stage were cultured to differentiate embryos in MS liquid medium supplemented with 0.5 mg/l BAP and 1 mg/l IAA for 20-30 days, followed by subculture in a semi-solid SH medium having the same growth regulators. Androgenesis has also been reported in five cultivars of lemon (*C. limon*) (Germana et al., 1990) and, recently, in *Citrus clementina* Hort. ex Tanaka and *C. reticulata* (Germana et al., 1994).

Cryopreservation of nucellar callus and somatic embryos was shown to be possible with the cultivar 'Washington' navel (*C. sinensis*). Viability of plant material was not affected by most of the treatments (Kobayashi et al., 1990; Marín et al., 1993)

Amo-Marco and Picazo (1994) used callus from *Citrus* fruit explants to study some aspects of fruit physiology. Albedo tissue from fruits of *C. sinensis* cv. 'Washington' navel of different age was used as the source of explants for calli induction. The authors concluded that growth (fresh weight) of the calli was dependent on the age of the fruit from which explants were taken, as well as on the addition of orange juice to the plant growth medium. Orange juice stimulated the growth of the calli and this growth was greater as the fruit age increased. The highest values were obtained when the albedo was isolated from fruits 30 to 45 mm in diameter and grown on medium supplemented with 20% orange juice.

Protoplast Isolation and Culture, and Plant Regeneration in *Citrus*

One of the first prerequisites to be successful in developing new somatic hybrids through protoplast fusion is the development of efficient protocols for protoplast isolation, fusion, and regeneration of somatic hybrid plants. Regeneration of plants from protoplasts, through⁻ tissue culture, is based on the theory of plant cell totipotency, proposed in the beginning of the century, which states that all totipotent cells have the genetic capacity to be converted to whole plants (Haberlandt, 1902).

The first successful plant protoplast isolation was done by Cocking (1960), and since then, protoplasts from numerous species have been isolated through enzymatic digestion and

successfully cultured. Early protocols consisted of mechanical isolation procedures, which normally provided variable yields and low reproducibility between successive isolations.

A series of literature reviews have reported the great amount of work dedicated to protoplast isolation, culture, and plant regeneration from many species. Among these reviews, the following can be cited: Litz and Gray (1992); Ochatt (1993); Ochatt et al. (1992); and Roest and Gilissen (1989, 1993). Specific reviews on *Citrus* protoplasts have been done by Gmitter et al. (1992); Grosser (1993, 1994), Grosser and Gmitter (1992), Louzada and Grosser (1994), and Vardi and Galun (1988, 1989). General reviews on somatic embryogenesis have been done by Jong et al. (1993) and Zimmerman (1993).

Kochba et al. (1972) described the first system by which ovule-derived nucellar callus of 'Shamouti' and 'Valencia' sweet oranges (*C. sinensis*) and 'Marsh' Seedless grapefruit (*C. paradisi*) could be regenerated into embryos and plants. Based on this system, subsequent experiments were performed, resulting in the successful isolation and culture of protoplasts from 'Shamouti' sweet orange nucellar callus, followed by embryo regeneration and plant recovery (Galun et al., 1977; Vardi, 1977; Vardi et al., 1975). As in other plant species, the division and further development of *Citrus* protoplasts was manifested only beyond a certain plating density (10^5 protoplasts/ml). However, Vardi and Raveh (1976) demonstrated that sparse plating could be compensated by

plating over a layer of division-arrested (X-irradiated) protoplasts from either *Citrus* callus or *Nicotiana* leaf mesophyll. A few years later, *Citrus* plant regeneration from protoplasts was successful and extended to include other *Citrus* varieties and one hybrid: 'Duncan' grapefruit (*C. paradisi*), sour orange (*C. aurantium*), 'Villafranca' lemon (*C. limon*), 'Dancy' and 'Ponkan' mandarins (*C. reticulata*), and 'Murcott' tangor (*C. reticulata* x *C. sinensis*) (Vardi, 1981; Vardi et al., 1982).

Hidaka and Kajiura (1988) also demonstrated plant regeneration from 'Ponkan' mandarin (*C. reticulata*) protoplasts and expanded the list of *Citrus* species and varieties regenerated from protoplasts with 'Washington' navel orange (*C. sinensis*) and Yuko (*C. yuko*). Sim et al. (1988) regenerated plants from protoplasts isolated from embryogenic suspension cultures of *Citrus mitis* Blanco.

Kobayashi et al. (1983) reported plant regeneration from 'Trovia' orange (*C. sinensis*) protoplasts isolated from embryogenic nucellar callus. Protoplasts were cultured in MT basal medium containing 0.15 M sucrose, 0.45 M glucose, and 0.6% agar, but without any plant growth regulators. In another report, the conditions of high frequency embryogenesis from 'Trovia' orange were described as being a combination of low cell densities (approx. $4 \times 10^4/\text{ml}$) and low mannitol concentration (approx. 0.4 M). Two alternatives to achieve high frequency embryogenesis (approx. 70%) were to either

dilute the cells to lower densities, or to do serial transfers of cells to fresh medium (Kobayashi et al. (1985).

Citrus plants regenerated from protoplasts have been very uniform in general morphology and genetic constitution. Kobayashi (1987), using 25 plants (protoplasts) regenerated from 'Trovita' orange (*C. sinensis*) protoplasts, evaluated several characters such as leaf and flower morphology, leaf oil, isozyme patterns, and chromosome number. No significant variations in any character were observed among the protoplasts, all of which were identical to nucellar seedlings.

Recently, other investigations have been carried out and allowed plant regeneration from protoplasts of additional *Citrus* species, such as Calamondin (*C. madurensis*) (Ling et al., 1989), and satsuma mandarin (*C. unshiu*) (Kunitake et al., 1991; Ling et al., 1990).

Niedz (1993) reported the effect of calcium alginate beads on plant regeneration of 'Hamlin' sweet orange from suspension culture-derived protoplasts. Plating efficiency exceeded 90% for Ca-alginate embedded protoplasts compared to 30% for protoplasts cultured in a liquid medium without Ca-alginate. The dissolution of the Ca-alginate matrix with a calcium sequestrant led to the formation of somatic embryos from protoplasts after 20 days. Embryos readily formed shoots that were rooted on MS + 0.01 μ M NAA + 5% sucrose.

Somatic Hybridization of Citrus Via Protoplast Fusion

A large number of *Citrus* somatic hybrids have been produced during the past 10 years. Protoplasts can be fused by either chemical or electronic methods (Grosser and Gmitter, 1990a), but the majority of the somatic hybrids produced so far have been obtained by chemical methods using polyethylene glycol (PEG). Tables 2, 3, and 4 summarize all interspecific and intergeneric *Citrus* somatic hybrids produced by PEG-induced protoplast fusion.

The first example of somatic hybridization involving *Citrus* was reported by Ohgawara et al. (1985), who described the production of an intergeneric allotetraploid hybrid from the fusion of embryogenic callus-derived protoplasts of 'Trovita' orange (*C. sinensis*) and mesophyll protoplasts of *P. trifoliata*, a sexually compatible *Citrus* related species. All the somatic hybrids reported since then have been produced by the fusion of embryogenic culture-derived protoplasts of the first parent with leaf mesophyll protoplasts of the second parent. A year later, Grosser (1986) reported the successful hybridization of 'Hamlin' sweet orange (*C. sinensis*) and *P. trifoliata*, cv. Flying Dragon, using embryogenic suspension culture-derived protoplasts of 'Hamlin' and leaf-derived protoplasts of Flying Dragon. In the same report, Grosser mentioned the production of another *Citrus* somatic hybrid with the fusion of embryogenic cell suspension-derived protoplasts of 'Hamlin' sweet orange and leaf mesophyll protoplasts of

Table 2: Interspecific *Citrus* somatic hybrids produced via PEG-induced protoplast fusion.

Combination	Reference
'Washington' navel orange [<i>Citrus sinensis</i> (L.) Osbeck] + 'Hayashi' satsuma mandarin (<i>Citrus unshiu</i> Marc.)	Kobayashi et al., 1988b
'Washington' navel orange (<i>C. sinensis</i>) + 'Murcott' tangor (<i>C. reticulata</i> x <i>C. sinensis</i>)	Kobayashi et al., 1988; Kobayashi and Ohgawara, 1988a
'Trovita' orange (<i>C. sinensis</i>) + 'Hayashi' satsuma mandarin (<i>C. unshiu</i>)	Kobayashi and Ohgawara, 1988
'Bahia' navel orange (<i>C. sinensis</i>) + 'Marsh' grapefruit (<i>C. paradisi</i>)	Ohgawara et al., 1989
'Key' lime (<i>C. aurantifolia</i>) + 'Valencia' sweet orange (<i>C. sinensis</i>)	Grosser et al., 1989
'Valencia' sweet orange (<i>C. sinensis</i>) + 'Femminello' lemon (<i>C. limon</i>)	Tusa et al., 1990
'Hamlin' sweet orange (<i>C. sinensis</i>) + 'Femminello' lemon (<i>C. limon</i>)	Tusa et al., 1992
Milam lemon (<i>C. jambhiri</i> hybrid) + 'Femminello' lemon (<i>C. limon</i>)	Tusa et al., 1992
Cleopatra mandarin (<i>C. reticulata</i>) + sour orange (<i>C. aurantium</i>)	Louzada et al., 1992
Cleopatra mandarin (<i>C. reticulata</i>) + rough lemon (<i>C. jambhiri</i>)	Louzada et al., 1992
Cleopatra mandarin (<i>C. reticulata</i>) + Volkamer lemon (<i>C. volkameriana</i>)	Louzada et al., 1992
Cleopatra mandarin (<i>C. reticulata</i>) + Rangpur lime (<i>C. limonia</i>)	Louzada et al., 1992
'Hamlin' sweet orange (<i>C. sinensis</i>) + Rangpur lime (<i>C. limonia</i>)	Louzada et al., 1992

Table 2--continued

Combination	Reference
sour orange (<i>C. aurantium</i>) + Volkamer lemon (zygotic seedling) (<i>C. volkameriana</i>)	Louzada et al., 1992
Smooth Flat Seville (<i>C. aurantium</i> hybrid) + rough lemon (<i>C. jambhiri</i>)	Louzada et al., 1992
'Valencia' sweet orange (<i>C. sinensis</i>) + Carrizo citrange (<i>C. paradisi</i> x <i>P. trifoliata</i>)	Louzada et al., 1992
'Hamlin' sweet orange (<i>C. sinensis</i>) + rough lemon (<i>C. jambhiri</i>)	Grosser et al., 1992b
'Valencia' sweet orange (<i>C. sinensis</i>) + rough lemon (<i>C. jambhiri</i>)	Grosser et al., 1992b
'Thompson' grapefruit (<i>C. paradisi</i>) + 'Murcott' tangor (<i>C. reticulata</i> x <i>C. sinensis</i>)	Grosser et al., 1992b
'Nova' mandarin-tangelo [<i>C. reticulata</i> x (<i>C. reticulata</i> x <i>C. paradisi</i>)] + 'Succari' sweet orange (<i>C. sinensis</i>)	Grosser et al., 1992a
'Hamlin' sweet orange (<i>C. sinensis</i>) + 'Dancy' tangerine (<i>C. reticulata</i>)	Grosser et al., 1992a
sour orange (<i>C. aurantium</i>) + Rangpur lime (<i>C. limonia</i>)	Grosser et al., 1994 -
Milam lemon (<i>C. jambhiri</i> x <i>C. sinensis</i>) + Sun Chu Sha mandarin (<i>C. reticulata</i>)	Grosser et al., 1994

Table 2: Intergeneric *Citrus* somatic hybrids, with sexually compatible parents, produced via PEG-induced protoplast fusion.

Combination	Reference
'Trovita' orange [<i>Citrus sinensis</i> (L.) Osbeck] + <i>P. trifoliata</i>	Ohgawara et al., 1985
'Hamlin' sweet orange [<i>Citrus sinensis</i> (L.) Osbeck] + <i>P. trifoliata</i> cv. Flying Dragon	Grosser, 1986; Grosser et al., 1988a
'Trovita' orange (<i>C. sinensis</i>) + Troyer citrange [<i>Citrus sinensis</i> (L.) Osbeck x <i>Poncirus trifoliata</i> (L.) Raf.]	Kobayashi and Ohgawara, 1988
'Bahia' navel orange (<i>C. sinensis</i>) + Troyer citrange (<i>C. sinensis</i> x <i>P. trifoliata</i>)	Ohgawara et al., 1991
Cleopatra mandarin (<i>C. reticulata</i>) + <i>P. trifoliata</i> cv. Flying Dragon	Grosser et al., 1992b
Cleopatra mandarin (<i>C. reticulata</i>) + Swingle citrumelo (<i>C. paradisi</i> x <i>P. trifoliata</i>)	Grosser et al., 1992b
'Meiwa' kumquat (<i>F. crassifolia</i>) + 'Valencia' sweet orange (<i>C. sinensis</i>)	Deng et al., 1992
Cleopatra mandarin (<i>C. reticulata</i>) + <i>P. trifoliata</i> cv. Argentine	Grosser et al., 1994
'Succari' sweet orange (<i>C. sinensis</i>) + <i>P. trifoliata</i> cv. Argentine	Grosser et al., 1994
sour orange (<i>C. aurantium</i>) + <i>P. trifoliata</i> cv. Flying Dragon	Grosser et al., 1994

Table 4: Intergeneric *Citrus* somatic hybrids, with sexually incompatible parents, produced via PEG-induced protoplast fusion.

Combination	Reference
'Hamlin' sweet orange (<i>C. sinensis</i>) + <i>S. disticha</i>	Grosser, 1986; Grosser et al., 1988b
'Hamlin' sweet orange (<i>C. sinensis</i>) + <i>S. buxifolia</i>	Grosser and Gmitter, 1990a, Grosser et al., 1992b
'Hamlin' sweet orange (<i>C. sinensis</i>) + <i>C. gillettiana</i>	Grosser and Gmitter, 1990a, b
Cleopatra mandarin (<i>C. reticulata</i>) + <i>C. gillettiana</i>	Grosser and Gmitter, 1990a; Grosser et al., 1990
'Hamlin' sweet orange (<i>C. sinensis</i>) + <i>A. ceylanica</i>	Louzada et al., 1993

Severinia disticha (Blanco) Swing.. This is the first example of a somatic hybrid between sexually incompatible woody species.

A few years later, Kobayashi and his colleagues reported the production of several *Citrus* somatic hybrids between sexually compatible species. The first hybrid was the result of the fusion of embryogenic suspension culture-derived protoplasts of 'Washington' navel orange (*C. sinensis*) with leaf-derived protoplasts of 'Hayashi' satsuma mandarin (*C. unshiu*) (Kobayashi et al., 1988b). Kobayashi and Ohgawara, (1988) and Kobayashi et al. (1988a) reported the production of three more *Citrus* somatic hybrids: 'Washington' navel orange (*C. sinensis*) plus 'Murcott' tangor (a probable sexual hybrid between *C. reticulata* and *C. sinensis*); 'Trovita' orange (*C. sinensis*) plus 'Hayashi' satsuma mandarin (*C. unshiu*); and 'Trovita' orange plus Troyer citrange (*C. sinensis* x *P. trifoliata*). Ohgawara's lab also produced somatic hybrids of 'Bahia' navel orange (*C. sinensis*) and 'Marsh' grapefruit (*C. paradisi*) (Ohgawara et al., 1989), and 'Bahia' navel orange (*C. sinensis*) with Troyer citrange (*C. sinensis* x *P. trifoliata*) (Ohgawara et al., 1991).

Grosser and his colleagues have also reported the production of more than 83 unique *Citrus* somatic hybrids in the past seven years (J.W. Grosser, personal communication). In 1988, they reported the successful somatic hybridization between the sexually incompatible species 'Hamlin' sweet

orange (*C. sinensis*) and *S. disticha* (Grosser et al., 1988b). In another paper, they reported the production of the somatic hybrid between the sexual compatible species 'Hamlin' sweet orange and *P. trifoliata*, cv. Flying Dragon (Grosser et al., 1988a). Both of these hybrids had been mentioned in the 1986 paper (Grosser, 1986), and are more extensively described in 1988.

In 1989, Grosser and his co-workers reported another successful interspecific somatic hybridization from the fusion of embryogenic suspension culture-derived protoplasts of 'Key' lime (*C. aurantifolia*) with leaf mesophyll-derived protoplasts of 'Valencia' sweet orange (*C. sinensis*) (Grosser et al., 1989). The expected characteristics of this new hybrid included good fruit quality, tristeza tolerance, and lime anthracnose resistance from 'Valencia', and a shortening of the juvenility period from 'Key' lime, which normally begins to bear fruits at around 18 months of age. Recently, trees from this fusion have produced fruit (J.W. Grosser, personal communication).

One year later, Tusa et al. (1990) reported another somatic hybrid between sweet orange and lemon. Embryogenic cell suspension cultures derived protoplasts of 'Valencia' sweet orange (*C. sinensis*) were fused with leaf mesophyll-derived protoplasts of 'Femminello' lemon (*C. limon*) resulting in the regeneration of somatic hybrid plants, autotetraploid lemon plants, and diploid plants from both parents. The

regeneration of plants from lemon leaf protoplasts was an example of protoplast-to-plant regeneration from non-nucellus-derived tissue of *Citrus*. The authors emphasized that the tetraploid lemon and somatic hybrid plants, if fertile, could be used in interploid sexual crosses to breed triploid seedless lemon cultivars with potential tolerance to mal secco disease from sweet orange.

Also in 1990, Grosser and colleagues reported several other intergeneric somatic hybrids, with sexually incompatible species. These included 'Hamlin' sweet orange (*C. sinensis*) plus *S. buxifolia* (Grosser and Gmitter, 1990a); 'Hamlin' sweet orange + *C. gillettiana* (Grosser and Gmitter, 1990a, b), and Cleopatra mandarin (*C. reticulata*) + *C. gillettiana* (Grosser and Gmitter, 1990a; Grosser et al., 1990). In the case of the somatic hybrid 'Hamlin' sweet orange + *C. gillettiana*, protoplasts from both leaf and non-embryogenic callus from the second parent were used in different fusions. However, recovery of somatic embryos was much less efficient from fusions involving *Citropsis* callus protoplasts than with leaf protoplasts (Grosser and Gmitter, 1990b).

In 1992, Grosser and his colleagues reported the production of 19 more *Citrus* somatic hybrids. Eight of these hybrids are plants with potential for use as rootstock varieties with improved disease resistance (Louzada et al., 1992). These hybrids include Cleopatra mandarin (*C. reticulata*) + sour orange (*C. aurantium*); Cleopatra mandarin

+ rough lemon (*C. jambhiri*); Cleopatra mandarin + Volkamer lemon (*C. volkameriana*); Cleopatra mandarin + Rangpur lime (*C. limonia*); 'Hamlin' sweet orange (*C. sinensis*) + Rangpur lime; sour orange + Volkamer lemon (zygotic seedling); 'Smooth Flat Seville' (*C. aurantium* hybrid) + rough lemon; and 'Valencia' sweet orange (*C. sinensis*) + Carrizo citrange (*C. paradisi* x *P. trifoliata*). Diploid plants were regenerated from nonfused callus-derived protoplasts of 'Valencia' sweet orange and Smooth Flat Seville and from nonfused leaf protoplasts of sour orange, Rangpur lime, rough lemon, and Volkamer lemon.

Grosser et al. (1992b) reported the production of six new somatic *Citrus* hybrids with potential for cultivar improvement. These combinations include 'Hamlin' sweet orange (*C. sinensis*) + *S. buxifolia* [already briefly mentioned by Grosser and Gmitter (1990a)]; Cleopatra mandarin (*C. reticulata*) + *P. trifoliata* cv. Flying Dragon; Cleopatra mandarin + Swingle citrumelo (*C. paradisi* x *P. trifoliata*); 'Hamlin' sweet orange + rough lemon (*C. jambhiri*); 'Valencia' sweet orange (*C. sinensis*) + rough lemon; and 'Thompson' grapefruit (*C. paradisi*) + 'Murcott' tangor. Besides the regeneration of somatic hybrid plants, the authors also reported the recovery of diploid plants from nonfused embryogenic culture-derived protoplasts of Cleopatra mandarin, and 'Hamlin' and 'Valencia' sweet orange, and from non-fused, leaf-derived protoplasts of rough lemon and 'Murcott' tangor. An interesting finding by the authors was that the hybrid

'Hamlin' + *S. buxifolia* had $2n=3x=27$, instead of the expected $2n=4x=36$ chromosomes.

In the same year, 1992, three more somatic hybrids with potential application in seedless cultivar development were reported by Grosser's group. Allotetraploid somatic hybrid plants of 'Nova' mandarin-tangelo [*C. reticulata* x (*C. reticulata* x *C. paradisi*)] + 'Succari' sweet orange (*C. sinensis*); and 'Hamlin' sweet orange (*C. sinensis*) + 'Dancy' tangerine (*C. reticulata*) were regenerated following protoplast fusion. Diploid plants were regenerated from unfused protoplasts of 'Hamlin', 'Nova', and 'Succari'. Tetraploid plants of 'Hamlin' and 'Succari' were also recovered, apparently resulting from homokaryotic fusions (Grosser et al., 1992a). Another report described the production of an intergeneric somatic hybrid for scion improvement from protoplast fusion of *F. crassifolia* cultivar 'Meiwa' with 'Valencia' sweet orange (*C. sinensis*) (Deng et al., 1992).

Tusa et al. (1992) completed the list of 19⁻ somatic hybrids produced by Grosser's group in that year, with the report of two more successful combinations, 'Hamlin' sweet orange (*C. sinensis*) + 'Femminello' lemon (*C. limon*), and Milam lemon (*C. jambhiri* hybrid) + 'Femminello' lemon (*C. limon*).

Louzada et al. (1993) reported somatic hybridization of *Citrus* with the sexually incompatible species *A. ceylanica*, by

fusing leaf mesophyll-derived protoplasts from this species with embryogenic callus-derived protoplasts of 'Hamlin' sweet orange. Two different types of leaf morphology were observed among the hybrids (normal and narrow) although no differences in chromosome number nor isozyme banding patterns were observed. This was the first report of the production of hybrids plants between these two genera.

Recently, Grosser et al. (1994) reported the production of five new *Citrus* somatic hybrids that potentially can be used as rootstocks due to their complementary characteristics. These combinations include Cleopatra mandarin (*C. reticulata*) + *P. trifoliata* cv. Argentine, 'Succari' sweet orange + *P. trifoliata* cv. Argentine, sour orange (*C. aurantium*) + *P. trifoliata* cv. Flying Dragon, sour orange + Rangpur lime (*C. limonia*), and Milam lemon [purported sexual hybrid of *C. jambhiri* x *C. sinensis*] + Sun Chu Sha mandarin (*C. reticulata*).

All the reports of somatic hybridization mentioned so far have dealt with chemically induced protoplast fusion by polyethylene glycol. Electrofusion methods have been rarely used and few somatic hybrids have been recovered so far. Saito et al. (1991) have reported the production of an acid *Citrus* somatic hybrid between 'Sudachi' (*C. sudachi*) and lime (*C. aurantifolia*), by electrofusion. The authors recovered 12 clones of a total of 40 embryos. Eight of the regenerated clones were shown to be somatic hybrids on the basis of

chromosome number and analysis of nucellar ribosomal DNA. The other 4 were recognized to have lime-type characteristics.

Hidaka and Omura (1992) reported the successful somatic hybridization between 'Saruwatari' satsuma mandarin (*C. unshiu*) and rough lemon (*C. jambhiri*), and 'Saruwatari' satsuma mandarin and Yuzu (*C. junos*), by electrofusion. The genomes from both parents were confirmed to be present in each combination by ribosomal rDNA analysis.

Shinozaki et al. (1992) tried to produce somatic hybrids of *Citrus* with the wild relative *M. paniculata*, by electrically-induced protoplast fusion. Embryogenic callus-derived protoplasts were fused electrically with leaf-derived protoplasts of the wild relative. The fusion efficiency (rates of binuclear heterokaryons) was around 15%. Only abnormal plantlets in vitro could be recovered, but they were verified by rDNA analysis, as somatic hybrids.

In the same way, only abnormal plantlets in vitro were regenerated when Takayanagi et al. (1992) attempted to produce *Citrus* somatic hybrids by electrofusion. The authors tried the combinations Mexican lime (*C. aurantifolia*) + Java feroniella [*Feroniella lucida* (Scheff.) Swing.] and Mexican lime + *S. glutinosa*. The abnormal plantlets were confirmed to be somatic hybrids by rDNA analysis.

Recently, Ling and Iwamasa (1994) reported the first successful intergeneric hybridization in woody species using electrofusion. Protoplasts of 'Ponkan' mandarin (*C.*

reticulata) and *Citropsis gabunensis* (Engl.) Swing. & M. Kell., both derived from embryogenic callus lines, were used in the experiment. Maximum fusion frequency was obtained with AC at 75 kV/cm (1.0 MHz) for 15 s, followed by DC square-wave pulses at 1.25 kV/cm for 40 μ s. Fusion treated protoplasts were cultured on MT medium containing no growth regulators, solidified with 0.6 Bacto Difco agar. Protoplast-derived calli were proliferated on MT medium containing 1 mg/l zeatin and 0.9% agar. A total of 31 lines of somatic hybrid calli were obtained by screening on the basis of chromosome count and isozyme analysis. The somatic hybrids were tetraploid ($2n=36$). Plants were regenerated from the calli via somatic embryogenesis. The somatic hybrid plants exhibited morphological characteristics intermediate to the parental plants.

The production of *Citrus* cybrids by donor-recipient protoplast fusions have also been reported in the literature. In fusions to produce cybrid plants, nuclear divisions of the donor protoplasts are arrested by X- or gamma-irradiation and the division of non-fused recipient protoplasts is inhibited by an antimetabolite such as iodoacetate, causing transient metabolic inhibition. Cybrids have been identified by analyzing mitochondrial DNA (mtDNA) profiles. Plastome analysis has also been utilized to identify and confirm the production of *Citrus* cybrids.

Green et al. (1986) constructed a physical plastome map for *C. aurantium*, and the plastomes of species and cultivars of *Citrus* and of two *Citrus* relatives were analyzed by Southern blot-hybridization of labelled total tobacco cpDNA to digests of *Citrus* DNA. The results showed some resemblance between plastomes of cultivars of lemon (*C. limon*), orange (*C. sinensis*), sour orange (*C. aurantium*), grapefruit (*C. paradisi*), and pummelo (*C. grandis*). The plastomes of mandarin (*C. reticulata*) and citron (*C. medica*) differed from the other species mentioned. *P. trifoliata* and *Microcitrus* sp. plastomes also appear to be distinct from the others, as well as those from the genus *Citrus*.

Kobayashi et al. (1991) analysed *Citrus* cytoplasmic genomes in the somatic hybrid of navel orange + 'Murcott' tangor. The 16 clones produced by protoplast fusion contained either one parental chloroplast genome or the other, but not both. In all cases, the mitochondrial genomes of the regenerated somatic hybrids were of the navel orange type.

Vardi et al. (1987) reported the first *Citrus*-cybrids produced by protoplast fusion. Successful plant recovery was obtained from fusions of 'Poorman' x *P. trifoliata* (donor) with 'Villafranca' lemon (*C. limon*) (recipient), 'Poorman' x *P. trifoliata* (donor) with sour orange (*C. aurantium*) (recipient), sour orange (donor) with 'Villafranca' lemon (recipient). Mitochondrial DNA restriction profiles were utilized for verification of the cybrids.

Two years later, Vardi and her colleagues reported two other *Citrus* cybrids produced by protoplast fusion. These were a *Microcitrus* sp. (donor) with rough lemon (*C. jambhiri*) (recipient); and a *Microcitrus* sp. (donor) with sour orange (*C. aurantium*) (recipient) (Vardi et al., 1989). These two intergeneric fusions resulted in novel mitochondrial DNA forms, indicating recombination between the chondriomes of *Citrus* and *Microcitrus*. Analyses of chloroplast DNA in fusion-derived embryos indicated that they contained the chloroplasts of either fusion-partner, or a mix of these chloroplasts. Later analysis of leaves from fully differentiated plants showed that cybrids with rough lemon morphology had either rough lemon or *Microcitrus* chloroplast DNA, but not both, indicating complete sorting out of chloroplasts. Likewise, sorting out of *Microcitrus* chloroplasts was detected in a cybrid plant having sour orange morphology (Vardi et al., 1989).

Recently, an interesting report of Saito and colleagues mentioned the production of one more *Citrus* cybrid (Saito et al., 1993). Through cell fusion between nucellar callus and mesophyll derived-protoplasts of two *Citrus* combinations, the authors recovered plants resembling mesophyll parents, besides the somatic hybrid plants. In two separate experiments involving the fusion of nucellar callus derived-protoplasts of 'Sudachi' (*C. sudachi*) and mesophyll protoplasts of lime (*C. aurantifolia*), 8 out of 18 clones regenerated were similar to

the mesophyll parent. Chromosome counting showed these plants to be diploid ($2n=2x=18$). The nuclear rDNA fragment patterns also was the same as those of mesophyll parents. However, the mtDNA analysis revealed that all of the clones that resembled mesophyll parents had mtDNA fragment patterns identical to those of the nucellar callus parent, indicating that these clones were cybrids. The authors suggested, based on these results, that the mitochondria of nucellar-derived callus cells may play a significant role in *Citrus* embryogenesis.

Genetic Transformation of *Citrus*

The introduction of foreign genes into orange protoplasts by direct DNA transfer has been reported by Kobayashi and Uchimaya (1989) by treating the protoplasts with a bacterial plasmid DNA carrying a chimeric gene. However, only ten microcallus colonies were obtained, of which eight survived and were subsequently grown on non-selective medium (antibiotic free medium), and only callus was obtained with no plant regeneration. Southern blot analysis showed the presence of the introduced DNA in one of the surviving colonies.

Vardi et al. (1990) reported the genetic transformation of *Citrus* protoplasts and regeneration of transgenic plants. The plasmid pCAP212 DNA, harboring the coding sequences of neomycin phosphotransferase (*npII*) and chloramphenicol acetyltransferase (*cat*) genes, was introduced successfully into rough lemon protoplasts via polyethylene glycol (PEG) treatment. Transient expression of *cat* activity was detected

3 days after the direct transformation was performed. Microcolonies were selected on agar medium containing paromycin sulfate (PAR). Twenty-one resistant clones, presumably containing the *nptII* gene, were isolated. Nine stably-transformed embryogenic clones were obtained, and two of these were regenerated into transgenic rooted plants. The transgenic nature of the clones was verified either through *nptII* activity or Southern hybridization.

Schell (1991) also reported successful regeneration of transformed plants from sweet orange protoplasts. 'Hamlin' sweet orange protoplasts were transformed by PEG-mediated direct DNA uptake using plasmid pcB10-106 containing a bacterial resistance gene for spectinomycin at 100 µg/ml, as well as the *GusA* and *NPTII* genes as scoreable markers. Putatively transformed plants were recovered, and transformation was confirmed by histochemical assay and polymerase chain reaction.

Hidaka et al. (1990a, b) reported *Agrobacterium*-mediated transformation in *Citrus*. Suspension cultures initiated from embryogenic callus lines of 'Trovita' and 'Washington' navel orange were used. Cell colonies were suspended in liquid medium and inoculated with an engineered *Agrobacterium* strain at a ratio of approximately 100 to 200 bacteria/cell colony. *NptII* and *hpt* (hygromycin phosphotransferase) chimeric genes, both with a cauliflower mosaic virus 35S promoter were tested.

At least one plant that differentiated from a hygromycin-resistant embryo was confirmed via Southern analysis.

Moore et al. (1992, 1993) also achieved successful transformation of *Citrus* by *Agrobacterium*. The authors developed an alternative method for transformation, involving internodal stem segments co-cultured with the *Agrobacterium*, followed by organogenic regeneration of shoots. Transformation and stable integration of a foreign gene in Carrizo citrange was confirmed by Southern blot analysis.

Recently, another successful transformation was reported with *P. trifoliata*. Kaneyoshi et al. (1994) developed a gene transfer system for trifoliolate orange using epicotyl segments infected with *Agrobacterium* harboring the binary vector pBI121 or pBI101-012-p1. Both vectors contained the neomycin phosphotransferase II (NPTII) and the β -glucuronidase (GUS) genes. In the plasmid pBI101-012-p1, the GUS gene was directed to the promoter region of ORF12 (rolC) of the Ri plasmid. On a selection medium containing 100 or 200 μ g/ml kanamycin, adventitious shoots were formed from 21.7-44.6% of the segments. The GUS gene was expressed in 55.4-87.7% of the shoots, as revealed by histochemical GUS assay. The stable integration of this gene was also confirmed by polymerase chain reaction (PCR) analysis and by Southern blot analysis. When the pBI101-012-p1 plasmid was used, the GUS activity was found to be located in phloem cells of leaf, stem and root.

Within 2-3 months, more than 100 transformed plants were obtained using this method.

Concluding Remarks

In conclusion, *Citrus* relatives have been described and tested in rootstock experiments, but their real value may be as germplasm resources for breeding and rootstock improvement, rather than direct use as rootstocks. Among the limitations of their use are the lack of good characterization and evaluation of this germplasm for tolerance or resistance to biotic and abiotic factors, important to the citrus industry, and their sexual incompatibility with *Citrus*.

Past and current literature indicates that biotechnologies (such as tissue culture, plant regeneration from protoplasts, and somatic hybridization through protoplast fusion) may be powerful tools to be used and combined with conventional breeding techniques in *Citrus* cultivar improvement programs. The use of these techniques may help to bring good traits from the *Citrus* relatives into the *Citrus* gene pool through somatic hybridization. The accomplishment of this objective will certainly provide a great contribution to *Citrus* cultivar improvement programs, increasing the diversity of the *Citrus* gene pool, and provide justification for resources spent to maintain germplasm collections.

MATERIALS AND METHODS

Experiments Involving the Search for Alternative Sources of Protoplasts of *Citrus* Relatives

Plant Material

Thirty selections, including 27 *Citrus* relatives maintained in a greenhouse at the Citrus Research and Education Center, UF-IFAS, Lake Alfred, were evaluated for non-embryogenic callus induction on 5 different media. An attempt was made to isolate protoplasts from some resulting callus lines, and protoplasts yields of selected species were determined. The selections used in this experiment are listed in Table 5.

Tissue Collection, Decontamination and Culture

Leaves and stems (and thorns when available) of all 30 selections (Table 5) were surface sterilized according to the method of Grosser and Chandler (1987) and cultured on 2 different non-embryogenic callus induction media (MTC and MTNC, Table 6). Three other media were also used for recalcitrant species (MTC and MTNC supplemented with 0.5 g/liter neutralized activated charcoal and MTL2 containing the synthetic auxin Picloram, Table 6). The surface sterilization of the explants had to be adapted for each selection to avoid

chlorine damage to the explants. In general, decontamination was obtained by immersion in 1N HCl for 1 to 5 sec, followed by 12 to 15 minutes in 1% sodium hypochlorite containing 2 drops of Liquinox detergent, followed by three 10-minute washes in sterile double-distilled water.

Explants were cultured in disposable Petri dishes (100 X 15 mm) (approximately 5 explants in each Petri dish), sealed with Nescofilm (Karlson Chemical), and maintained in the dark at 28 °C. Explants and the initial calli were transferred to new media approximately every 6 weeks.

Qualitative evaluations were conducted every month and calli were classified into 5 categories, based on their quality and vigor as follows:

- | | |
|------------|---------------------------|
| (1) [-] | No callus induction |
| (2) [+] | Slow growing, hard callus |
| (3) [++] | Fast growing, hard callus |
| (4) [+++] | Slow growing, soft callus |
| (5) [++++] | Fast growing, soft callus |
-

Observations of root induction were also noted. The best growing calli were selected from each selection in an effort to establish long-term callus lines. The best results for protoplast isolation from non-embryogenic callus have been obtained from soft callus followed by fast-growing hard callus (J.W. Grosser, unpublished data).

Approximately 6 months after culture initiation, 10 selections that exhibited the best callus quality and vigor were selected for protoplast isolation. For each selection, the following 2 treatments were used (Grosser and Gmitter, 1990a):

- (1) Approximately 1.0 g fresh callus + 2.5 ml 0.6 M BH3 + 1.5 ml enzyme solution
- (2) Approximately 1.0 g fresh callus + 2.5 ml 0.7 M BH3 + 1.5 ml enzyme solution

Qualitative evaluation of the protoplast yield for each species was conducted by approximating the percentage of tissue digestion and/or protoplast liberation. Protoplast yield was classified into three categories according to cell separation and/or protoplast liberation: Low ($\leq 33\%$ cell separation and/or protoplast liberation), medium (33-67% cell separation and/or protoplast liberation), high ($\geq 67\%$ cell separation and/or protoplast liberation).

Experiments Involving Somatic Hybridization of Citrus Via Protoplast Fusion with Emphasis on Wide Hybridization with Citrus Relatives

Plant Material

Plant material from different sources was used for protoplast isolation in the present research. Material of selected embryogenic parents was selected from a collection of ovule-derived embryogenic callus and ovule-derived embryogenic suspension cultures, maintained at the Citrus Research and

Table 5: Taxonomic identification of *Citrus* and its relatives used in the callus induction experiment.

-
- 1) *Aegle marmelos* (L.)
 - 2) *Afraegle gabonensis* (Swing.) Engl.
 - 3) *Afraegle paniculata* (Schum.) Engl.
 - 4) *Atalantia ceylanica* (Arn.) Oliv.
 - 5) *Atalantia monophylla* DC.
 - 6) *Balsamocitrus dawei* Stapf.
 - 7) *Citropsis gillettiana* Swing. & M.Kell.
 - 8) *Citrus ichangensis* Swing. (sub-genus *Papeda*)
 - 9) *Citrus jambhiri* Lush. (rough lemon)
 - 10) *Clausena lansium* (Lour.) Skeels
 - 11) *Eremocitrus glauca* (Lindl.) Swing.
 - 12) *Faustrimedim* [*Microcitrus australasica* (F.Muell.) Swing.
X (*Fortunella* sp. (Swingle) X *Citrus reticulata*
'Calamondin' (Blanco))]
 - 13) *Fortunella hindsii* (Champ.) Swing.
 - 14) *Glycosmis pentaphylla* (Retz) Corrêa
 - 15) *Citrus grandis* (L.) Osbeck
 - 16) *Hesperethusa crenulata* (Roxb.) Roem.
 - 17) Lemonine [Purported hybrid of *Citrus limon* (L.) Burm. f.
and *Citrus aurantifolia* (Christm.) Swing.]
 - 18) *Microcitrus australis* (Planch.) Swing.
 - 19) *Microcitrus australasica* (F.Muell.) Swing.
 - 20) *Microcitrus* (hybrid)
 - 21) *Microcitrus papuana*
 - 22) *Murraya paniculata* (L.) Jack
 - 23) *Pamburus missionis* (Wt.) Swingle
 - 24) *Poncirus trifoliata* (L.) Raf. cv. Flying Dragon
 - 25) *Poncirus trifoliata* (L.) Raf. cv. Argentine
 - 26) Procimequat [(*Citrus aurantifolia* (Christ.) Swing. cv.
Mexican X *Fortunella japonica* (Thumb.) Swing.) X
Fortunella hindsii (Champ.) Swing.]
 - 27) *Severinia buxifolia* (Poir.) Tenore -
 - 28) *Severinia disticha* (Blanco) Swing.
 - 29) *Swinglea glutinosa* (Blanco) Merr.
 - 30) *Zanthoxylum fagara*
-

Table 6: Growth regulators used with MT basal medium (Murashige and Tucker, 1969) for the induction and maintenance of non-embryogenic callus (mg/liter).

Growth regulators ²	Media		
	MTC ^y	MTNC ^y	MTL2
2,4-D	0.663	---	---
Kinetin	0.22	0.22	---
NAA	---	5.60	---
6-BAP	---	---	0.01
Picloram	---	---	0.06
Coconut water (ml/l)	20.0	20.0	20.0

Media pH = 5.8

² 2,4-D = 2,4-dichlorophenoxyacetic acid; NAA = Napthalene acetic acid; 6-BAP = 6-benzylaminopurine.

^y These media tried with and without 0.5 g/liter neutralized activated charcoal.

Table 7: Taxonomic identification of *Citrus* relatives used in protoplast fusion experiments.

<i>Aegle marmelos</i> (L.)	
<i>Afraegle gabonensis</i> (Swing.) Engl.	
<i>Afraegle paniculata</i> (Schum.) Engl.	
<i>Atalantia ceylanica</i> (Arn.) Oliv.	
<i>Atalantia monophylla</i> DC.	-
<i>Atalantia</i> sp.	
<i>Balsamocitrus dawei</i> Stapf.	
<i>Citropsis gillettiana</i> Swing. & M.Kell.	
<i>Clausena lansium</i> (Lour.) Skeels	
<i>Feronia limonia</i> (L.) Swing.	
<i>Murraya paniculata</i> (L.) Jack	
<i>Pamburus missionis</i> (Wt.) Swingle	
<i>Severinia buxifolia</i> (Poir.) Tenore	
<i>Severinia disticha</i> (Blanco) Swing.	
<i>Swinglea glutinosa</i> (Blanco) Merr.	

Table 8: Taxonomic identification of *Citrus* species and cultivars used in protoplast fusion experiments.

Scientific name	Common name/cultivar
<i>Citrus aurantifolia</i> (Christm.) Swing.	'Key' lime
<i>Citrus aurantium</i> (L.)	Sour orange
<i>Citrus aurantium</i> (L.) x <i>Citrus reticulata</i> Blanco or <i>Citrus grandis</i> (L.) Osbeck x <i>C. reticulata</i> (purported) hybrid	Natsudaaidai hybrid
<i>Citrus grandis</i> (L.) Osbeck x [<i>Citrus sinensis</i> (L.) Osbeck x <i>Citrus aurantium</i> (L.)] (purported)	'Smooth Flat Seville'
<i>Citrus ichangensis</i> Swing.	Ichang papeda
<i>Citrus limon</i> (L.) Burm. f.	'L'Apireno Continnella' lemon ('LAC' lemon)
<i>Citrus paradisi</i> Macf. x <i>Citrus reticulata</i> Blanco	'Minneola' tangelo
<i>Citrus reticulata</i> Blanco	Changsha mandarin
<i>Citrus reticulata</i> Blanco	'Dancy' tangerine
<i>Citrus reticulata</i> Blanco	'Ponkan' mandarin
<i>Citrus reticulata</i> Blanco	Sun Chu Sha mandarin
<i>Citrus reticulata</i> Blanco x (<i>Citrus paradisi</i> Macf. x <i>C. reticulata</i>)	'Nova' mandarin-tangelo
<i>Citrus reticulata</i> Blanco x (<i>Citrus paradisi</i> Macf. x <i>C. reticulata</i>)	'Page' tangelo

Table 8--continued

Scientific name	Common name/cultivar
<i>Citrus reticulata</i> X <i>Citrus sinensis</i> (purported)	'Murcott' tangor
<i>Citrus reticulata</i> Blanco	Cleopatra mandarin
<i>Citrus sinensis</i> (L.) Osbeck	'5215' navel orange
<i>Citrus sinensis</i> (L.) Osbeck	'Russ' navel orange
<i>Citrus sinensis</i> (L.) Osbeck	'Hamlin' sweet orange
<i>Citrus sinensis</i> (L.) Osbeck	'Rohde Red Valencia' sweet orange
<i>Citrus sinensis</i> (L.) Osbeck	'Succari' sweet orange
<i>Citrus sinensis</i> (L.) Osbeck	'Valencia' sweet orange
<i>Citrus sinensis</i> (L.) Osbeck x <i>Poncirus trifoliata</i> (L.) Raf.	Carrizo citrange

Education Center, UF-IFAS, Lake Alfred. Material of the non-embryogenic parent was obtained from leaves of plants maintained in a greenhouse or seedlings in a growth chamber. Other sources of non-embryogenic parents used included leaf-derived protoplasts from seedlings germinated in vitro and from some non-embryogenic callus lines obtained from a different experiment, described in a later chapter.

Tables 5 and 6 summarize all the genera, species and varieties utilized in protoplast fusion experiments. Table 7

gives the scientific name of all *Citrus* relatives studied in the present protoplast fusion research, and Table 8 summarizes the taxonomic identification of *Citrus* species and cultivars used in such experiments, with the respective common name. In order to be consistent regarding the citation of such species and varieties, all *Citrus* species will be referred as their common name in the next chapters. Please refer to Tables 3, 5 and 6 for complete taxonomic classification.

Protoplast Isolation

Composition of the enzyme solution used for protoplast isolation in most experiments of this research is described in the Appendix. This composition was adjusted in some cases in order to "fine-tune" for genotypic and epigenetic differences.

Embryogenic suspension cultures

The suspensions were maintained without any growth regulators, in 40-50 ml of liquid EME or H+H (see Appendix), in 125 ml Erlenmeyer flasks covered with aluminum foil, sealed with masking tape. Cultures were maintained on a shaker at 140 rpm with no supplemental lighting. Suspensions were subcultured every two weeks by splitting the contents between two sterile flasks and adding a half volume (25 ml) of fresh culture medium. The cells were used for protoplast isolation 5-14 days after subculture.

Two variations in the protoplast isolation protocol from embryogenic suspension cultures were used (Grosser and

Gmitter, 1990a). For well established, vigorous suspensions, 2 ml of suspension (4-12 days into the 2-weeks subculture cycle) were transferred to a 60 mm X 15 mm Petri dish (usually 2 to 3 plates for each suspension were used), with a widemouth pipet. Two and a half ml of enzyme solution were added, drop by drop, with a Pasteur pipet to facilitate cell separation and enzyme penetration. Petri dishes were sealed with Nescofilm and incubated 6-16 hours at 28 °C on a rotary shaker at 50 rpm.

For vigorously growing young embryogenic suspensions, 1-2 ml of suspension (4-12 days old on a 2 week subculture cycle) were transferred into a 60 mm X 15 mm Petri dish with a widemouth pipet. The liquid suspension culture medium was carefully removed with a Pasteur pipet and replaced with 3 ml 0.6 BH3 (see Appendix). After that, 1.0-1.5 ml enzyme solution was added, drop by drop. The Petri dishes were sealed with Nescofilm and incubated as previously described.

Callus cultures

Protoplasts can be readily isolated from friable embryogenic, ovule-derived callus maintained on growth-regulator-free media and from friable, non-embryogenic seedling or young-tree organ derived callus, originated and maintained on media containing high levels of auxin (Grosser and Gmitter, 1990a; Grosser et al., 1988b). Callus culture conditions were a temperature of 28 °C and light intensity approximately 30 $\mu\text{M PAR}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ (PAR=photosynthetic active

radiation). Protoplast isolation was achieved by gentle mechanical maceration of about 1 g (fresh weight) friable callus (2-3 weeks old on a 4-6 week subculture cycle) into 2.5-3.0 ml of 0.7 M BH3 medium in 60 X 15 mm Petri dish, followed by the addition of 1-1.5 ml enzyme solution, drop by drop. Petri dishes were sealed with Nescofilm and incubated as previously described.

For non-friable callus, normally those from non-embryogenic cultures, the enzyme solution was increased to 2.0-2.5 ml per Petri dish in order to obtain better penetration and protoplast yield.

Leaves

Most of the leaf material utilized for protoplast isolation in this research was obtained from seedlings and/or young plants grown in a growth chamber or in a greenhouse at CREC-Lake Alfred, which required prior decontamination before protoplast isolation.

Decontamination of selected leaves from vigorous growing plants was achieved by immersion in 1 N HCl for 1-3 seconds followed by 12-15 minutes immersion in 1% sodium hypochlorite containing three drops Liquinox soap or other surfactant, followed by a 5 minute rinse in double-distilled H₂O. Damaged vascular tissue was dissected with a sharp scalpel and two more 15 minute rinses in double-distilled water were performed. Leaves were then feathered or cut into thin strips with a sharp scalpel and incubated in a 11 ml total volume of

enzyme solution and BH3, normally 0.6 BH3 (3:8 v:v ratio of enzyme solution:BH3 medium for very tender tissue and 4:7 v:v ratio for more hardened tissue). Side-arm flasks, 125 ml, with side arms covered with a double-layer of miracloth (Cal Biochem) to prevent contamination were used. Flasks containing leaves were then evacuated for 12 to 15 minutes at 50 KPa to facilitate enzyme infiltration and incubated as previously described.

Leaf material from in vitro-grown nucellar seedlings also was used for some parents when available. This is considered the best source of leaf material for fusion experiments, because this eliminates the need for decontamination prior to isolation (Grosser and Gmitter, 1990a). The procedure utilized for obtaining in vitro grown seedlings is described as follows. Mature fruits were immersed in 70% ethanol and flamed. Fruits were cross-sectioned in a sterile, laminar air flow hood, and the seeds were aseptically removed and placed on RMAN medium (see Appendix). Germination occurred within 2-3 weeks, and the leaves produced could be used for protoplast isolation without the need to be decontaminated.

Other adjustments in the general protocol were also made according to the plant material for best results, mostly involving the collection methods of the leaves from the greenhouse. These adjustments were as follows. For those plants maintained in the greenhouse, the best time of the day for leaf material collection proved to be early in the

morning, 2-3 hours after sunrise, probably because less water and temperature stresses occurred at this time. Leaves collected at later times during the day showed considerably greater amount of tissue damage during the decontamination procedure. Also, in order to reduce the amount of open stomata, leaves collected from plants in the greenhouse were immediately placed in zip-lock bags, which were filled with air (to slightly increase CO₂ concentration) and placed in the dark for at least 15 minutes prior to decontamination. All these precautions were taken in order to reduce tissue damage as much as possible during the decontamination process and to maximize protoplast yield.

Leaf age was also critical in some cases. For most donors, young mature leaves were the best tissue for protoplast isolation, but for species such as *P. missionis*, leaves just prior to full maturity gave the best results for protoplast isolation and yield, particularly when 1.0 g of resin (Amberlite XAD-7 [Sigma]) was added to the enzyme cocktail to prevent damage from phenolics and other compounds released during digestion.

Protoplast Purification

After incubation, all protoplast preparations were purified on a sucrose-mannitol gradient (Grosser and Gmitter, 1990a), described as follows: Protoplast preparations were first passed through a 45 µm stainless steel or nylon mesh screen to remove undigested cell clumps and debris into 15 ml

calibrated screw-top centrifuge tubes. The material was then centrifuged at 100 g for 4-10 minutes. The variation in time was based on the volume of material. The supernatant was removed with a Pasteur pipet, and the pellet was gently resuspended in 5 ml CPW medium containing 25% sucrose (see Appendix); 2 ml of CPW medium containing 13% mannitol was slowly pipetted directly on top of the sucrose layer, avoiding any mixing of the two layers. The tubes were centrifuged for 6 minutes at 100 g. Viable protoplasts usually collect in a band at the interface between the two layers. These cells were removed with a Pasteur pipet and resuspended in appropriate amount of BH3 medium in another centrifuge tube (see Appendix). Purified protoplasts were then ready for further manipulation.

Protoplast Fusion

Protoplasts can readily be fused by either chemical or electronic methods (Grosser and Gmitter, 1990a). The chemical method using polyethylene glycol (PEG) has been used extensively at Grosser's lab, because it has been shown to be simple, efficient, inexpensive, and does not seem to interfere with protoplast viability.

According to research done so far, protoplasts from one of the parental sources must have a capacity for embryogenesis as a requirement for plant regeneration following protoplast fusion. Normally, embryogenic callus-derived or embryogenic suspension culture derived protoplasts are fused with leaf-

derived or other non-totipotent protoplasts (like those derived from non-embryogenic callus).

The procedure described as follows has been adapted from Menczel et al. (1981) by Grosser and Gmitter (1990a). Approximately equal volumes of purified protoplasts from each parental source were mixed in BH3 medium and centrifuged for 4 minutes at 100 g. The pelleted, mixed protoplasts were resuspended in a total volume of BH3 medium equal to 10X to 20X the volume of the original pellet. Two drops of the resuspended mixture were pipeted into the center of a Petri dish (60 mm X 15 mm). The number of Petri dishes was dependent on the volume of resuspended mixed protoplasts. Two drops of PEG solution (see Appendix) was added immediately to each Petri dish and incubated for 8 minutes. It is very important that the PEG solution is fresh, because it is not well buffered, and it acidifies over time. Previous research at Grosser's lab indicated that old batches of PEG will not reduce fusion frequency but may have a negative effect on protoplast viability following fusion. After the protoplasts have been incubated with PEG, two drops of A + B elution solution (9:1 v:v, see Appendix) were added to each fusion dish at the side of the shallow liquid pool at the center of each dish. After another incubation of 12 minutes, 12 drops of fresh BH3 medium were added around the periphery of the fusing protoplasts. Following another incubation of 5 minutes, PEG + elution solution were carefully and slowly removed with a

Pasteur pipet, and immediately replaced with 15 drops of BH3 medium. After 10 minutes, the medium was carefully removed and replaced with another 12-15 drops of fresh BH3 medium. This step was repeated two more times, with great care to avoid removing protoplasts from the fusion Petri dishes.

After the final wash, protoplasts were cultured directly in the fusion Petri dishes by adding the 4-8 drops of protoplast culture medium in a shallow pool in the center of the petri dish. Several drops of fresh protoplast culture medium were placed around the perimeter to maintain high humidity. Fusion plates were incubated in darkness at 28 °C.

Three different protoplast culture media were selected and used for protoplast culture after fusion. These media were BH3 (0.6 M), EME (0.6 M), and a mixture of BH3/EME (1:1 v:v). Previous research, done by several authors, has indicated that the carbon/osmoticum source (sugar and sugar alcohols) used in various *Citrus* protoplast culture media has played a major role on protoplast plating efficiency (defined as the percentage of protoplasts that divide to produce ⁻callus colonies), and plant regeneration.

Protoplast Culture

First cell divisions were usually observed 10-14 days after protoplast fusion and plating (Grosser and Gmitter, 1990a). Plating efficiency generally ranges from 0 to 35% for straight protoplast culture. Cultures were supplemented after 5-6 weeks (4 weeks for more vigorous colonies) with fresh

liquid medium containing reduced osmoticum. This medium consisted of 10-12 drops of a 1:1:1 (v:v:v) mixture of BH3 medium:EME (0.6 M):EME medium (0.145 M, or regular EME) (Appendix).

Following another incubation period of 2-4 weeks in low light intensity (approximately $10 \mu\text{M PAR.m}^{-2}.\text{s}^{-1}$), further reduction of osmoticum was accomplished by adding 1-2 ml of a 1:2 (v:v) mixture of BH3 medium:regular EME per plate. At this point, vigorous cultures were transferred (by pouring) to 100 mm x 15 mm Petri dishes containing agar-solidified EME medium. All other liquid culture plates were supplemented with 10-12 drops of liquid regular EME every 2 weeks until adequate growth allowed transfer to solid medium, as previously described.

Plant Regeneration

Cultures that were transferred to solidified EME medium (by pouring) were kept moist with the previous liquid mixture, until callus colonies had adapted to the solid medium environment. Light was also supplied at a rate of approximately $10 \mu\text{M PAR.m}^{-2}.\text{s}^{-1}$.

For most combinations, somatic embryo formation occurred spontaneously, usually 6-16 weeks following the initial plating of protoplasts. For some combinations, embryo formation was never achieved. Embryogenesis also seemed to be influenced by callus vigor. If the cultures were extremely vigorous, no embryo formation was observed. Small embryos that

were produced were transferred to Petri dishes containing fresh solidified EME for initial development.

Following 4-6 weeks, developed embryos approximately 0.5 cm diameter in size were transferred to EME 1500 medium for enlargement. EME 1500 medium is the basic EME medium with 1500 mg/l malt extract added (see Appendix). Large, well shaped somatic embryos were germinated directly on B+ medium (see Appendix). Irregular embryos were cut and cultured on DBA3 for shoot proliferation and elongation (see Appendix). Shoots were transferred to rooting medium RMAN (see Appendix) in Magenta boxes. Light intensity was increased to approximately 70-100 $\mu\text{M PAR.m}^{-2}.\text{s}^{-1}$. Rooted plantlets were finally transferred to a commercial potting mixture containing 60% peat and 40% polybeads and maintained at high humidity, light intensity of approximately 70 $\mu\text{M PAR.m}^{-2}.\text{s}^{-1}$, and 28 °C until acclimated.

New treatments were tried in an attempt to induce embryogenesis and regenerate plants from some very important, but recalcitrant combinations. Different media for embryo initiation and for embryo germination were tested, in addition to the media used routinely in the lab for these purposes as previously mentioned.

In an effort to improve embryo induction, one small experiment with three media combinations was conducted, in which new media containing different growth regulators and/or carbon sources were tested. The different media tested were the normal EME medium [MT medium (Murashige and Tucker, 1969)

plus 500 mg/l malt extract] with the following modifications: Addition of cobalt ($25 \mu\text{M}$), or BA ($13.3 \mu\text{M}$), or the substitution of sucrose for glycerol (2.5%), or galactose (0.1 M) + sorbitol (0.1M). As a control, EME medium, normally utilized for embryo stimulation, was used. Three different fusion combinations were selected. These combinations were, 'Succari' + *P. missionis* (callus already cultured on solid EME), and colonies in small Petri dishes (before pouring onto EME) from the combinations 'Succari' + *M. paniculata*, and 'Succari' + *A. gabonensis*. This material was evaluated for embryo induction and embryo quality.

Improvements for embryo development and germination were also attempted. 500 mg/l activated charcoal was added to 1500 germination medium, and brown embryos that were found in some combinations were cultured on this medium. BGN medium (Louzada et al., 1993) was also tested in combinations with *A. ceylanica*, and WPM medium (Russell and McCown, 1986) was tried for combinations involving *C. lansium*, which had shown some yellowish embryos. WPM contains NH_4NO_3 at 4.94 mM and $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ at 2.35 mM as an alternative nitrogen source. For the combinations involving *B. dawei*, *C. lansium*, and *M. paniculata*, other media were tested in addition to DBA3 which was routinely used. These media include DBA3 with activated charcoal (500 mg/l), CW28 (with and without activated charcoal, at the same concentration of 500 mg/l), and CW32 (with and without activated charcoal). CW28 composition is the

basic MT medium with the addition of the following plant growth regulators: malt extract (500 mg/l), BAP (500 mg/l), IBA (250 mg/l), adenine (30 mg/l), GA₃ (1 mg/l) (Wan, 1989). CW32 composition is the basic MT medium with the addition of the following plant growth regulators: malt extract (500 mg/l), BAP (500 mg/l), IBA (250 mg/l), adenine (30 mg/l), coumarin (10 mg/l), GA₃ (1 mg/l) (Wan, 1989). For complete description of these media please see Appendix.

Hybrid Verification

Characteristics expected of true somatic hybrids include vegetative morphology intermediate to the donor parents, additive tetraploid chromosome numbers, and composite expression of DNA or gene product markers (Grosser and Gmitter, 1990a). It is important to emphasize that none of these methods alone provide sufficient confirmation, but rather each is subject to limitations and can give positive results from situations other than somatic hybridity (Grosser and Gmitter, 1990a), so it is essential that these methods be applied together in hybrid verification.

Morphological evaluation

Citrus and related genera present morphological differences that greatly facilitate somatic hybrid identification (Grosser and Gmitter, 1990a). Traits under the control of dominant or co-dominant genes are usually easily identifiable in somatic hybrid plants, particularly in wide

combinations. Also, according to Grosser and Gmitter (1990a), somatic hybrid plants can usually be identified by the expression of any trait or traits unique to the non-embryogenic parent. Examples that illustrate this include expression of the trifoliate leaf character from *Poncirus* in *Citrus* + *Poncirus* somatic hybrid plants (Grosser et al., 1988a; Ohgawara et al., 1985), expression of red pigment in new flush from *Severinia* in *Citrus* + *Severinia* somatic hybrid plants (Grosser et al., 1988b), and partial expression of the pentafoolate leaf character from *Citropsis* in *Citrus* + *Citropsis* hybrid plants (Grosser and Gmitter, 1990b).

The use of morphological differences to identify interspecific somatic hybrids plants in *Citrus* can be much more difficult than in the case of intergeneric hybrids (Grosser and Gmitter, 1990a), but more subtle differences in characteristics such as leaf petiole wing size and leaf blade shape and thickness may be useful to identify putative somatic hybrid plants. Normally, interspecific somatic hybrid plants have a petiole wing size intermediate to that of the parents, and the increased ploidy level of interspecific somatic hybrid plants can result in increased leaf thickness and a darker green leaf color (Grosser and Gmitter, 1990a). The identity of selected putative somatic hybrid plants was further confirmed by cytogenetic or molecular verification procedures.

Chromosome number determinations

Chromosome number determinations of somatic hybrid plants were carried out for all putative somatic hybrid plants regenerated by protoplast fusion experiments. The hematoxylin staining technique described by Grosser and Gmitter (1990a), and briefly described below was used for mitotic preparations.

Active root tips were excised 3-4 hours after the onset of light and placed in a supersaturated aqueous solution of 1-4 dichlorobenzene at 4 °C for 2 hours. Special care was taken in the selection of root tips, in that preference was given to those very active and white in color. After incubation in 1-4 dichlorobenzene root tips were rinsed in double-distilled H₂O and transferred to a 3:1 (v:v) solution of 100% ethanol:acetic acid for 2 hours. After this step, root tips could be stored overnight in 70% ethanol for further manipulations or could be directly hydrolyzed in 5 N HCl for 20 minutes. Following a rinse in double-distilled H₂O, root tips were transferred to a 4% FeNH₄(SO₄)₂ solution for 2 hours, thoroughly washed in double-distilled H₂O for at least 30 minutes (normally 90 minutes), and stained in a solution of 0.2% hematoxylin for 30 minutes or more. After staining, root tips were rinsed in double-distilled H₂O, squashed with a smooth, round tipped glass rod onto a glass slide in 45% acetic acid solution, spread under a cover slip, and examined. Preparations were scanned for metaphase cells at 200X magnification, and

chromosomes were counted at 600X, or 1000X magnification under oil immersion.

Molecular markers analyses

The verification of the hybridity of the recovered plants was performed by at least one molecular marker analysis.

Leaf isozymes. For some plants, electrophoretic analysis of leaf isozymes was performed. Isozyme banding patterns of crude leaf tissue extracts from the putative somatic hybrids and their parental genotypes were developed on horizontal mixed starch (9.85%) and agarose (0.15%) gels with histidine-citrate buffer (pH 5.7). Electrophoresis was carried out for 3 hours at 4 °C at 60 mA constant current. The gel was sliced and stained for peroxidase (*Per*), phosphoglucumutase (*Pgm-1*), and phosphoglucose isomerase (*Pgi*) as described by Vallejos, 1983.

RAPD analysis. Isozyme analysis has been a useful tool for distinguishing a large number of *Citrus* interspecific and intergeneric sexual and somatic hybrids (Torres et al., 1978; Grosser and Gmitter, 1990a). However, isozyme analysis in *Citrus* has also presented some disadvantages, and maybe the most important limitation of its use resides in its low level of polymorphisms, i.e., the number of isozyme alleles is very limited in *Citrus*, and as a consequence, most intraspecific and some interspecific hybrids cannot be unambiguously distinguished from nucellar or parental types. Other molecular marker methods such as RFLP (Restriction Fragment Length

Polymorphism) analysis can also be used for hybrid identification. RFLP analysis can be a very accurate and direct method to verify the hybridity of various *Citrus* progeny including somatic hybrids (Kobayashi et al., 1991; Ohgawara et al., 1985), but, on the other hand, it is a very tedious, time-consuming, and expensive procedure (Xiao et al., personal communication).

Another technique called RAPD (random amplified polymorphic DNA) analysis has been used for hybrid identification. RAPD analysis was first proven to be a powerful tool for genome mapping in *Citrus* (Cai et al., 1994), and for taxonomic relationships and genome mapping in other plants (Liu and Furnier, 1993; Ragot and Hoisington 1993; Kennard et al., 1994; Landry et al., 1994; Novy et al., 1994; Thormann et al., 1994). Recently, RAPD markers were developed for confirmation of somatic hybrids in potato (Takemori et al., 1994).

More recently, RAPD analysis has been developed and adapted to distinguish *Citrus* zygotic seedlings from nucellar seedlings, and true somatic hybrids from regenerated parental types (Xiao et al., personal communication). As previously mentioned, one objective of the present research was to use and test this technique to identify most of the somatic hybrids produced in this project. For some recovered plants, DNA amplification was performed by polymerase chain reaction (PCR) with random decamer primers. The procedures used for DNA

extraction, and the reaction components and PCR conditions were those of Xiao et al. (personal communication); the methods are described below.

DNA extraction. Approximately 1-2 small shoot tips (100 mg or less) were placed into sterile 1.5 ml Eppendorf tubes and ground in liquid nitrogen with a toothpick for approximately 30 seconds. An aliquot of 700 μ l extraction buffer (100 mM Tris-HCl pH 8.0, 50 mM Na₂EDTA pH 8.0; 500 mM NaCl, 10 mM beta mercaptoethanol, 3% SDS) was added to the samples, followed by grinding for a few minutes. Tubes were left at room temperature until all samples were prepared. The samples were incubated in a 65 °C water bath for 10 min and centrifuged at 12,000 rpm, for 10 min. The supernatant was transferred to clean tubes, and an equal volume of phenol was added to each sample. Tubes were quickly inverted a few times and centrifuged at 12,000 rpm for 10 min. Aqueous phase was transferred to clean tubes, and an equal volume of chloroform was added to each. Tubes were inverted a few times and centrifuged again at 12,000 rpm, for 10 min. Aqueous phases were transferred to clean tubes with the addition of 0.1 volume of 7.5 M ammonium acetate and 0.6 volume of isopropanol. Tubes were centrifuged for 2 min, the supernatant was discarded and tubes washed with 75% ethanol; the pellets were dried in vacuum and dissolved in 100 μ l sterile H₂O. DNA concentrations were measured by spectrophotometry ($\lambda=260$), and

the DNA was diluted to 50 ng/ μ l accordingly. DNA samples were stored at -20 °C prior to use.

PCR amplification. Several random 10-mer primers were screened initially to compare the banding patterns of the two parents involved in the somatic hybridization. If some polymorphism was detected by a given primer, a new PCR reaction was performed with this primer, including lanes with the two parents and DNA from the putative hybrid. After this initial screening the following primers were selected: A-7, W-4, A-15, A-17, Q-20, U-14, V-02, and W-15 (Operon Technologies, Alameda, CA). PCR was conducted with an PTC-100 Programmable Thermal Controller (MJ Research, Watertown, MA), using the following program: 93 °C for 2 min (initial denaturation); then 1 min at 92 °C; 35 °C for 1 min; 72 °C for 2 min; for 43 cycles; and then 72 °C for 10 min. Each 15 μ l of reaction mixture contained 50 mM Tris-HCl pH 8.3, 250 μ g/ml BSA, 2% Ficoll, 1mM Tartazine, 2 mM MgCl_2 , 200 μ M dNTPs, 0.8 μ M primer, 1 unit of Taq polymerase (Promega), and 30-50 ng of genomic DNA. DNA amplification products were separated in 1.8% agarose gels with 1x TAE buffer and 25 ppm ethidium bromide. Electrophoresis was carried out for 2-3 hours at room temperature, at 150 mA constant current in 1x TAE buffer solution with 20 ppm ethidium bromide. Gels were examined and photographed under UV light.

RESULTS AND DISCUSSION

Experiments Involving the Search for Alternative Sources of Protoplasts of Citrus Relatives

Callus Induction and Quality

A qualitative evaluation of in vitro responses of the 30 selections is presented in Table 9. The different types of calli found and classified are represented in Figure 1a-d.

Best results were obtained from *C. gillettiana*, which produced calli that were soft and extremely fast growing. Further selections of calli from this species showed better performance on MTC than on MTNC. Soft, moderately fast-growing callus was obtained from *A. marmelos*, *A. gabonensis*, *A. paniculata*, *B. dawei*, *C. jambhiri*, and *C. grandis*. Soft, slow-growing callus was obtained from the *Microcitrus* hybrid and *Z. fagara*. Fast-growing hard calli were obtained on MTC and MTNC media from Faustrimedin, *F. hindsii*, *P. missionis*, *P. trifoliata* cvs. Flying Dragon and Argentine, Procimequat, *S. disticha*, and *S. buxifolia*.

Activated charcoal in MTC and MTNC seemed to enhance the performance of several callus lines by improving color and growth rate. MTL2 medium, used for a few selections that did not respond on any of the other media, was able to induce hard, slow-growing callus from Faustrimedin and *H. crenulata*.

No calli were induced on any media from *C. lansium*, *M. paniculata*, and *S. glutinosa*.

Root formation was observed at variable frequency from several selections (Table 10). Root induction was most often observed on MTNC medium and was apparently due to NAA, an auxin that has been used to promote rooting in *Citrus* (Sabbah et al., 1991).

Preliminary analysis of fresh cuts of soft and hard callus, and staining with phloroglucinol did not show any consistency for accumulation of more lignin in hard callus (data not shown). However, cells from hard callus tended to be more compact, small and irregular, as compared to cells from soft callus.

Protoplast Yield

Protoplasts were obtained from soft callus tissue of 7 of the 10 selections tested, although medium to high yields were obtained only from *C. gillettiana* and *C. jambhiri* (Table 11, Figure 1-f). No protoplasts were obtained from Lemonine, *S. disticha*, and *S. buxifolia*. The isolation of protoplasts from older friable-callus lines of *S. disticha* (Grosser et al., 1988b) and *S. buxifolia* (Grosser et al., 1992b) has been reported previously. As expected, soft, fast-growing calli were best for protoplast isolation. Similar results were obtained using 0.6M and 0.7M BH3 media in the enzyme preparation.

Table 9: Non-embryogenic callus induction in *Citrus* and its relatives (see Table 5 for complete taxonomic identification).

Species	Culture media				
	MTC	MTNC	MTCch ²	MTNCch ²	MTL2
<i>Aegle m.</i>	+++	++++	++	+++	N
<i>Afraegle g.</i>	+++	++++	+++	+++	N
<i>Afraegle p.</i>	+	++++	N	N	N
<i>Atalantia c.</i>	+++	+++	N	N	N
<i>Atalantia m.</i>	+	-	+	-	-
<i>Balsa. d.</i>	++	++++	++	++	N
<i>Citropsis g.</i>	++++	+++	N	N	N
<i>Citrus ichang.</i>	++	++	N	N	N
<i>Citrus jamb.</i>	++++	++	N	N	N
<i>Clausena l.</i>	-	-	-	-	-
<i>Eremocitrus</i>	+	++	-	+	-
<i>Faustriamedin</i>	++	++	-	++	+
<i>Fortunella</i>	++	++	N	N	N
<i>Glycosmis</i>	+	-	-	-	N
<i>Citrus grand.</i>	+++	+++	N	+++	N
<i>Hesperethusa</i>	+	-	-	-	+
<i>Lemonine</i>	+++	++	N	N	N
<i>Micro. a.</i>	++	++	+	++	N
<i>Micro. a.a.</i>	-	+	-	+	-
<i>Micro. hyb.</i>	+++	++	N	N	N
<i>Micro p.</i>	-	+	N	+	-
<i>Murraya p.</i>	-	-	-	-	-
<i>Pamburus</i>	++	++	++	N	N
<i>Poncirus FD</i>	++	++	++	N	N
<i>Poncirus A</i>	++	++	N	N	N
<i>Procimequat</i>	++	++	++	++	- N
<i>Severinia b.</i>	++	++	++	+	N
<i>Severinia d.</i>	++	++	N	N	N
<i>Swinglea g.</i>	-	-	-	-	-
<i>Zantho.</i>	+++	+++	N	N	N

²With activated charcoal.

N = not tested

- = no callus

+ = slow growing hard callus

++ = fast growing hard callus

+++ = slow growing soft callus

++++ = fast growing soft callus

Table 10: Selections exhibiting root formation.

Selection	Root frequency ²
<i>C. ichangensis</i>	High
<i>C. jambhiri</i>	Medium
<i>E. glauca</i>	Medium
Faustrimedin	Medium
<i>F. hindsii</i>	Medium
<i>C. grandis</i>	Low
Lemonine	Medium
<i>M. australis</i>	Low
<i>Microcitrus</i> hybrid	Low
<i>M. papuana</i>	Low
<i>P. trifoliata</i> cv. Flying Dragon	High
<i>P. trifoliata</i> cv. Argentine	High
Procimequat	High
<i>S. disticha</i>	Low

²Low = 1-2 roots/calli; Medium = 3-6 roots/calli; High = >6 roots/calli

Table 11: Approximated protoplast yield from callus tissue of 10 selected species.

Species	Yield ²
<i>A. marmelos</i>	Low
<i>A. gabonensis</i>	Low
<i>A. paniculata</i>	Low
<i>B. dawei</i>	Low
<i>C. gillettiana</i>	High
<i>C. grandis</i>	Low
Lemonine	No yield
<i>C. jambhiri</i>	Medium
<i>S. disticha</i>	No yield
<i>S. buxifolia</i>	No yield

²Low = 33% cell separation and/or protoplast liberation; Medium = 33-67% cell separation and/or protoplast liberation; High = >67% cell separation and/or protoplast liberation.

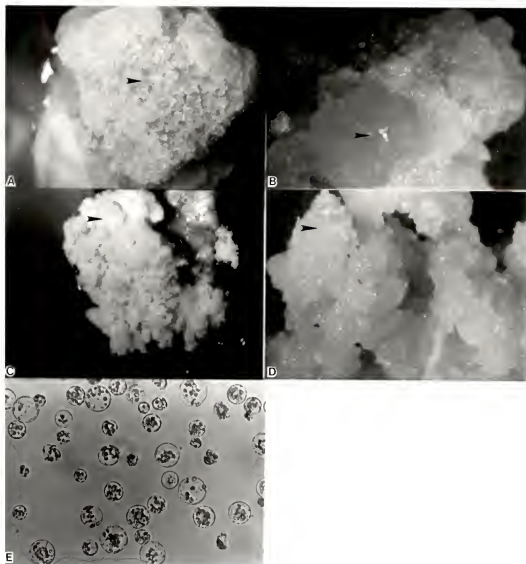


Figure 1: Representative calli and protoplasts from *Citrus* relatives: (a) slow-growing hard callus; (b) fast-growing hard callus; (c) slow-growing soft callus; (d) fast-growing soft callus; (e) protoplasts isolated from calli of *A. paniculata* (400x magnification).

Experiments Involving Somatic Hybridization of Citrus
Via Protoplast Fusion with Emphasis on Wide Hybridization
with Citrus Relatives

Summary of the Parental Combinations Used in Fusion
Experiments

Table 12 summarizes all the parental combinations chosen for fusion experiments in the present research. The first parent of each combination is the embryogenic donor, from which protoplasts were isolated from either nucellus-derived embryogenic callus lines or nucellus-derived embryogenic suspension cultures. Protoplasts from the second parent are either leaf-derived from plants grown in the greenhouse or seedlings grown in vitro, or from non-embryogenic callus.

As previously mentioned, the primary objective of this research was to develop a reliable system for the wide hybridization of *Citrus* via protoplast fusion. The search for alternative sources for protoplast isolation from the wild relatives, as well as the adaptation of the protocols already in use for protoplast fusion in *Citrus* to expand further the use of somatic hybridization in the subfamily Aurantioideae were also main goals of this research project.

The third objective of the present work was to produce somatic hybrids that would combine sweet orange with selected mandarins and mandarin hybrids (tangelos and tangors) at the tetraploid level, with an ultimate goal of producing improved seedless fresh fruit varieties with an expanded range of maturity dates and better storage characteristics. These

Table 12: Protoplast fusion combinations performed.

Date	Combination
02-25-92	Smooth Flat Seville + Carrizo citrange
03-20-92	'Succari' sweet orange + <i>P. missionis</i>
03-20-92	Cleopatra mandarin + <i>P. missionis</i>
03-20-92	'Nova' mandarin-tangelo + <i>A. marmelos</i>
03-26-92	'Nova' mandarin-tangelo + <i>A. marmelos</i>
03-31-92	'Nova' mandarin-tangelo + <i>S. buxifolia</i>
03-31-92	'Succari' sweet orange + <i>A. gabonensis</i>
04-01-92	'Nova' mandarin-tangelo + <i>A. gabonensis</i>
04-01-92	'Succari' sweet orange + <i>A. gabonensis</i>
04-01-92	'Succari' sweet orange + <i>S. buxifolia</i>
04-01-92	Cleopatra mandarin + <i>S. buxifolia</i>
04-01-92	'Nova' mandarin-tangelo + <i>S. buxifolia</i>
04-03-92	Cleopatra mandarin + <i>M. paniculata</i>
04-10-92	'Nova' mandarin-tangelo + <i>M. paniculata</i>
04-10-92	Cleopatra mandarin + <i>S. glutinosa</i>
04-14-92	'Succari' sweet orange + 'Ponkan' mandarin
04-14-92	'Succari' sweet orange + 'Page' tangelo
04-14-92	'Succari' sweet orange + 'Minneola' tangelo
04-14-92	'Succari' sweet orange + 'Dancy' tangerine
04-16-92	'Valencia' sweet orange + 'Ponkan' mandarin
04-16-92	'Valencia' sweet orange + 'Minneola' tangelo
04-16-92	'Succari' sweet orange + 'Ponkan' mandarin
04-16-92	'Valencia' sweet orange + 'Page' tangelo
04-23-92	'Succari' sweet orange + 'Dancy' tangerine
04-23-92	'Valencia' sweet orange + 'Dancy' tangerine
04-23-92	'Valencia' sweet orange + <i>C. gillettiana</i>
04-29-92	'Succari' sweet orange + <i>A. marmelos</i>
04-29-92	'Succari' sweet orange + <i>C. gillettiana</i>
05-01-92	'Succari' sweet orange + <i>A. marmelos</i>
05-01-92	'Succari' sweet orange + <i>C. gillettiana</i>
05-05-92	Cleopatra mandarin + <i>S. disticha</i>
05-05-92	'Hamlin' sweet orange + <i>S. disticha</i>
05-05-92	'Nova' mandarin-tangelo + <i>S. disticha</i>
05-05-92	Smooth Flat Seville + <i>S. disticha</i>
05-06-92	'Succari' sweet orange + <i>S. disticha</i>
05-06-92	'Nova' mandarin-tangelo + <i>S. disticha</i>
05-06-92	'Valencia' sweet orange + <i>S. disticha</i>
05-06-92	Cleopatra mandarin + <i>S. disticha</i>
05-12-92	'Valencia' sweet orange + <i>B. dawaiei</i>
05-12-92	'Nova' mandarin-tangelo + <i>B. dawaiei</i>
05-12-92	'Valencia' sweet orange + <i>S. glutinosa</i>
05-15-92	'Nova' mandarin-tangelo + <i>S. glutinosa</i>

Table 12--continued

Date	Combination
05-15-92	Cleopatra mandarin + <i>S. glutinosa</i>
05-15-92	'Nova' mandarin-tangelo + <i>B. dawaei</i>
05-15-92	Cleopatra mandarin + <i>B. dawaei</i>
05-15-92	Smooth Flat Seville + <i>P. missionis</i>
05-20-92	'Nova' mandarin-tangelo + <i>B. dawaei</i>
05-20-92	Cleopatra mandarin + <i>S. glutinosa</i>
05-20-92	'Nova' mandarin-tangelo + <i>S. glutinosa</i>
05-20-92	'Nova' mandarin-tangelo + <i>P. missionis</i>
05-20-92	Smooth Flat Seville + <i>B. dawaei</i>
05-20-92	Cleopatra mandarin + <i>B. dawaei</i>
05-20-92	'Valencia' sweet orange + <i>B. dawaei</i>
05-22-92	'Valencia' sweet orange + <i>P. missionis</i>
05-22-92	'Hamlin' sweet orange + <i>P. missionis</i>
05-29-92	Cleopatra mandarin + <i>C. lansium</i>
05-29-92	'Nova' mandarin-tangelo + <i>C. lansium</i>
07-16-92	Smooth Flat Seville + <i>C. gillettiana</i>
07-16-92	'Nova' mandarin-tangelo + <i>C. gillettiana</i>
07-16-92	'Nova' mandarin-tangelo + <i>C. lansium</i>
06-08-93	'Succari' sweet orange + <i>M. paniculata</i>
06-08-93	'Succari' sweet orange + <i>B. dawaei</i>
06-09-93	'Succari' sweet orange + <i>A. paniculata</i>
06-09-93	'Succari' sweet orange + <i>Atalantia</i> sp.
06-11-93	'Succari' sweet orange + <i>A. paniculata</i>
06-11-93	'Succari' sweet orange + <i>A. ceylanica</i>
06-11-93	'Succari' sweet orange + <i>B. dawaei</i>
06-16-93	'Succari' sweet orange + <i>A. ceylanica</i>
06-16-93	'Succari' sweet orange + <i>B. dawaei</i>
06-17-93	'Succari' sweet orange + <i>P. missionis</i>
06-17-93	'Succari' sweet orange + <i>C. lansium</i>
06-29-93	'5215' navel orange + <i>P. missionis</i>
06-29-93	'Murcott' tangor + <i>C. lansium</i>
06-29-93	'5215' navel orange + <i>A. gabonensis</i>
07-06-93	'LAC' lemon + <i>C. gillettiana</i>
07-06-93	'Key' lime + <i>C. gillettiana</i>
07-15-93	'5215' navel orange + <i>A. ceylanica</i>
07-27-93	'LAC' lemon + <i>A. monophylla</i>
07-27-93	'LAC' lemon + <i>C. gillettiana</i>
07-28-93	'Valencia' sweet orange + <i>P. missionis</i>
07-28-93	'Rohde Red Valencia' sweet orange + <i>P. missionis</i>
08-03-93	'Valencia' sweet orange + <i>P. missionis</i>
08-03-93	'Hamlin' sweet orange + <i>P. missionis</i>
08-03-93	'LAC' lemon + <i>B. dawaei</i>

Table 12--continued

Date	Combination
08-05-93	'Succari' sweet orange + <i>P. missionis</i>
08-05-93	'Murcott' tangor + <i>P. missionis</i>
08-05-93	'Murcott' tangor + <i>S. glutinosa</i>
08-10-93	'5215' navel orange + <i>P. missionis</i>
08-10-93	'Succari' sweet orange + <i>P. missionis</i>
08-10-93	'Nova' mandarin-tangelo + <i>B. dawei</i>
08-10-93	'Nova' mandarin-tangelo + <i>P. missionis</i>
08-13-93	'Succari' sweet orange + <i>P. missionis</i>
08-13-93	'Succari' sweet orange + <i>F. limonia</i> "93-01"
08-17-93	<i>S. glutinosa</i> '8604' + sour orange
08-17-93	'Succari' sweet orange + <i>F. limonia</i> "93-01"
08-19-93	<i>S. glutinosa</i> '8604' + Changsha mandarin
08-19-93	<i>S. glutinosa</i> '8604' + sour orange
08-19-93	Natsudaiddai hybrid + Sun Chu Sha mandarin
08-20-93	<i>S. glutinosa</i> '8604' + 'Succari' sweet orange
08-20-93	'Succari' sweet orange + <i>B. dawaei</i>
08-21-93	'Key' lime + <i>C. gillettiana</i>
09-01-93	'5215' navel orange + <i>B. dawaei</i>
09-13-93	'Nova' mandarin-tangelo + <i>C. lansium</i>
09-13-93	'5215' navel orange + <i>F. limonia</i> "93-02"
09-17-93	'Succari' sweet orange + <i>F. limonia</i> "93-01"
09-21-93	'Succari' sweet orange + <i>F. limonia</i> "93-01"
09-28-93	'Succari' sweet orange + <i>M. paniculata</i>
09-28-93	'5215' navel orange + <i>M. paniculata</i>
09-30-93	'Succari' sweet orange + <i>M. paniculata</i>
10-01-93	'Hamlin' sweet orange + <i>C. ichangensis</i>
10-01-93	'Succari' sweet orange + <i>C. ichangensis</i>
10-01-93	'LAC' lemon + <i>M. paniculata</i>
10-01-93	'5215' navel orange + <i>B. dawaei</i>
10-05-93	'Nova' mandarin-tangelo + <i>M. paniculata</i> -
10-05-93	'Dancy' tangerine + <i>M. paniculata</i>
10-07-93	'Murcott' tangor + <i>F. limonia</i> "93-01"
10-07-93	'Succari' sweet orange + <i>F. limonia</i> "93-01"
10-07-93	'Russ' navel orange + <i>F. limonia</i> "93-01"
10-15-93	'Succari' sweet orange + <i>C. ichangensis</i>
10-19-93	'Succari' sweet orange + <i>F. limonia</i> "93-02"
10-19-93	'LAC' lemon + <i>F. limonia</i> "93-02"
10-19-93	'Succari' sweet orange + <i>A. marmelos</i>
10-21-93	'Hamlin' sweet orange + <i>A. paniculata</i>
11-16-93	'Nova' mandarin-tangelo + 'Ponkan' mandarin
11-16-93	'LAC' lemon + 'Ponkan' mandarin

hybrids may serve as tetraploid breeding parents for use in interploidy crosses to produce seedless triploids.

Table 12 lists several fusion combinations performed with many different wild *Citrus* relatives. The table also lists several interspecific combinations attempted to combine sweet orange with mandarins/mandarin hybrids.

After a series of fusion experiments performed by mid-summer of 1992, the research was resumed in the summer of 1993 with focus on the most difficult fusion combinations, including intertribal combinations. Therefore, as a part of the objectives of this research work, more time and effort was invested in the isolation, fusion, and culture techniques for obtaining such wide hybrids, in an attempt to improve the technique already available for the isolation, fusion, and culture of the interspecific hybrids.

It is important to mention that most of these combinations, especially those performed after summer 1993, have been carefully chosen for a specific reason or because of a previous observation. 'Succari' sweet orange cell suspension culture became the preferred embryogenic parent utilized in the fusions because previous work had shown the excellent compatibility of this line with several wild relatives in fusions (by analyzing the data of fusions performed in the first tests period, spring 1992). Other reasons for the use of this line were: a) 'Succari' can transmit low acidity to the fruits of its progeny (Barrett, 1990); b) many of the original

intergeneric hybrids previously produced by Grosser and his colleagues were made with 'Hamlin' sweet orange, because of the availability and performance of the callus and suspension line. However, *Citrus* rootstocks are usually propagated by seed, which produce true-to-type plants because of nucellar polyembryony. 'Hamlin' sweet orange produces very few seeds and wide hybrids produced using 'Hamlin' may therefore not produce enough polyembryonic seed for efficient propagation. To increase the potential for polyembryonic seed production in intergeneric hybrids of *Citrus* with the more distant relatives, subsequent hybrids were made with the seedy 'Succari' sweet orange. Some other embryogenic parents were also utilized as an alternative.

Some of the wild relatives were chosen because of their potential for improving *Citrus*, and also because of their phylogenetic distance from the *Citrus* genus. Most of these genera such as *Pamburus*, *Afraegle*, *Balsamocitrus*, *Swinglea*, *Aegle*, and *Feronia* have never been reported in hybridization experiments with *Citrus*. Other selections chosen were *M. paniculata*, *A. ceylanica*, *C. gilletiana*, and *C. ichangensis*, this last one chosen because of its excellent cold tolerance.

Mandarin types ('Murcott' tangor and 'Nova') were tried as an alternative embryogenic parent for fusion with *C. lansium*, in addition to the 'Succari' embryogenic cell suspension. Observations of graft compatibility from the Tropical Research and Education Center (TREC), Homestead

(University of Florida) revealed good graft compatibility of *C. lansium* with mandarins.

The parents for fusion with *C. gillettiana* were also carefully chosen. The hybrid already produced by Grosser and Gmitter (1990b) ('Hamlin' sweet orange + *C. gillettiana*) has shown some splitting in the bark in the preliminary evaluation experiments. The problem might be overcome by choosing a more vigorous parent that exhibits continuous growth (versus distinct flushes). The more tropical varieties ('LAC' lemon and 'Key' lime) were therefore chosen for somatic hybridization with *C. gillettiana*.

As emphasized before, more effort was invested in developing and/or improving techniques for the isolation prior to fusion and culture of the protoplasts for many of these combinations, because most of them had never been attempted, and the standard technique already developed had not always been adequate for these combinations. Isolation of the protoplasts was carried out using leaves harvested early in the morning instead of mid afternoon, as had been the method of choice previously. Alterations in the molarity of the enzyme solution, and the use of resins for absorption of phenolic compounds liberated during the isolation, were also performed. The use of resins (Amberlite XAD-7) [Sigma]) improved results for protoplast isolation from leaves of *P. missionis*. The stage of development of the leaves was also

crucial for this species. The best results were obtained using immature, only slightly hardened leaves.

To provide another alternative source of protoplasts, fruits were collected from the wild *Citrus* relatives selected at Department of Plant Industry (DPI) *Citrus* collection in Winter Haven, FL, in July of 1993. Seeds were extracted under aseptic conditions and germinated in rooting medium (RMAN) utilized for plant regeneration in Magenta boxes (Magenta Corp., Chicago, IL). The species utilized were *A. paniculata*, *A. gabonensis*, *C. ichangensis*, *C. lansium*, and *P. missionis*. Some of these species did not germinate well, but the others germinated and produced seedlings with leaves well suited for protoplast isolation, such as *A. paniculata* and *C. lansium*. The main advantages of this source of protoplasts are that they do not require previous decontamination for isolation, and they provide consistently high protoplast yields (Grosser and Gmitter, 1990a).

Another source of protoplasts sometimes used was the non-embryogenic callus lines developed specifically for this research (discussed previously in this chapter). Some of these parents generated excellent callus (very vigorous and friable) and permitted the isolation of protoplasts of good quality. The combinations 'Succari' + *B. dawei*, on August 20, 1993, and 'Key' lime + *C. gillettiana*, on August 21, 1993, include protoplasts of the wild parent from this callus (Table 12). Unfortunately no colony development was observed after fusion

for these combinations. Some other callus lines have exhibited a hard consistency, which inhibited enzyme digestion. In an attempt to overcome this problem, the volume of the enzyme solution was increased to up to 3.0 ml per Petri dish in these cases, but with no success.

In the case of *S. glutinosa*, in addition to the normal source of leaves from greenhouse plants, the embryogenic callus line '8604' was also utilized. Some embryos were recovered from the combination with sour orange, but no plants were regenerated, even when the embryos were cultured in alternative media, such as CW28 and CW32.

As previously mentioned, several of the combinations attempted did not develop because of contamination or failed cell division or colony development after fusion. In other cases, cell division did occur, but after the colonies had been transferred to solid medium, embryogenesis was never achieved.

Embryogenesis also seemed to be affected by callus vigor. If the colonies were extremely vigorous, no embryo formation was observed. This is consistent with the results observed by Schell (1991).

In terms of the culture of the protoplasts after fusion, new treatments were tried in an attempt to induce embryogenesis and regenerate plants from some very important combinations. Different media for embryo initiation and for embryo germination were tried, in addition to the media

already used routinely in the lab for these purposes (EME, 1500, B+, DBA3 and RMAN) (Grosser and Gmitter, 1990a) (see Appendix).

For embryo induction three tests were performed, using new media containing different growth regulators and/or carbon sources, following some ideas in the literature (Ben-Hayyim and Neumann, 1983; Gavish et al., 1991; Kochba et al., 1978b, 1982; Singh, A.K. et al., 1992). The different media tested were the normal EME medium [MT medium (Murashige and Tucker, 1969) plus 500 mg/l malt extract] with the following modifications: Addition of cobalt (25 μ M), or BA (13.3 μ M), or the substitution of sucrose for glycerol (2.5%), or for galactose (0.1 M) + sorbitol (0.1M). As a control, EME medium, normally utilized for embryo stimulation, was used. As described earlier, three different fusion combinations were selected: 'Succari' + *P. missionis* (callus already cultured in EME), and colonies in small Petri dishes (before pouring onto EME) from 'Succari' + *M. paniculata*, and 'Succari' + *A. gabonensis*.

The total number of embryos observed in each treatment for each combination selected is shown in Table 13. Evaluations were performed 30, 90, and 120 days after treatment. More embryos were produced from EME + glycerol, EME + galactose + sorbitol, and EME (control), than from the other media.

However, all embryos observed were of very poor quality, with low vigor and aberrant morphological development. Other embryos showed vitrification and yellowish color. All embryos produced on these media were transferred to fresh EME and other culture media for development and germination.

Table 13: Total number of embryos counted in five different media for three selected protoplast fusion combinations, after 120 days.

Culture media	'Succari' + <i>P. missionis</i>	'Succari'+ <i>M. paniculata</i>	'Succari'+ <i>F. limonia</i>
EME	30	5	13
EME/Glycerol	57	7	26
EME/BA	16	2	9
EME/Cobalt	4	6	8
EME/Galactose/Sorbitol	40	8	31

Embryos from the combinations 'Succari' sweet orange + *M. paniculata* and 'Succari' sweet orange + *P. missionis* did not result in plant recovery, but, from the combination 'Succari' + *F. limonia*, numerous plants (more than 65) were regenerated from all treatments.

The 1500 medium with activated charcoal (500 mg/l) was tried with some combinations that produced brown embryos. BGN medium (Louzada et al., 1993) was tested in combinations with *A. ceylanica*, with no significant results. Fusions involving *C. lansium* produced several yellowish embryos. In an attempt

to test whether the nitrogen source played a role in embryo development of *C. lansium*, the yellowish embryos were transferred to WPM medium (Russell and McCown, 1986). WPM is a medium in which the ammonium nitrate concentration of MT medium was reduced and the nitrogen was also supplied as calcium nitrate, i.e., ammonium/nitrate ratio is reduced. For the combinations involving *B. dawei*, *C. lansium*, and *M. paniculata*, additional media were tested besides the DBA3 medium routinely used. These media were DBA3 with activated charcoal (500 mg/l), CW28 (with and without 500 mg/l activated charcoal), and CW32 (with and without activated charcoal) (see Appendix).

Combinations involving *A. ceylanica*, in particular those with 'Succari' sweet orange, exhibited no differences in embryo recovery and embryo quality when embryos were cultured on BGN medium. In fact, hybrids with adequate vigor could be regenerated on normal DBA3 medium, as verified by subsequent molecular marker analyses. No improvement in embryo development was achieved for combinations involving *B. dawaeei*, and *M. paniculata*, when embryos were cultured in the different media tested, even after more than 6 successive transfers to fresh medium, every 4-6 weeks. However, some weak shoots were produced by organogenesis when irregular embryos from the fusions 'Succari' sweet orange + *C. lansium*, and 'Murcott' tangor + *C. lansium* were cultured for more than 6 weeks in the medium CW-32, without activated charcoal. All the

shoots produced had pubescent stems, and leaves, which are morphological characteristics of *C. lansium*. These shoots, when placed in the routinely used rooting medium, RMAN, did not produce adventive roots. Some of these shoots were micrografted by the insertion of a 1.5 cm stem of the putative hybrid onto decapitated, in vitro-grown, etiolated, grapefruit or mandarin seedlings. Very few shoots survived micrografting and those that did developed into weak plants. The plants regenerated did not exhibit morphology of either 'Succari', 'Murcott', or *C. lansium*. RAPD analysis of the most vigorous plant from the combination 'Murcott' tanger + *C. lansium* did not confirm that it was a hybrid.

Several combinations involving 'LAC' lemon, in particular those with *C. gilletiana*, *F. limonia*, and *M. paniculata*, produced several hundred plants, but unfortunately, all these plants were shown to be lemon plants. This is the first case of massive plant regeneration with absolutely no apparent hybrids or cybrids, regardless of the second parent or culture sequence. There is no obvious reason why this happened, but possibly there may be some genetic mechanism in 'LAC' lemon that prevents it from producing somatic hybrids with other *Citrus* species/varieties and their relatives, or the correct selection scheme for the somatic hybrid has not been determined.

The 'Succari' + *A. paniculata* combination also produced about 70 plants that were acclimated to growth chamber

conditions. However, RAPD analysis revealed that those plants had genetic composition identical to sweet orange.

Another noteworthy fact is the high embryogenic capacity of the callus line '5215' navel orange observed in the fusion experiments. However, the embryos formed were extremely irregular and abnormal, regardless of the second fusion parent, and it was very difficult to induce germination and plant regeneration. This was the case for all the combinations involving this parent, in particular the navel + *A. gabonensis* and navel + *P. missionis* combinations.

Intergeneric Wide Hybrids Produced and their Potential for Citrus Rootstock Improvement

Table 14 shows all the intergeneric combinations that resulted in proven somatic hybrid plants, after transfer to soil. All hybrid plants had leaf morphology intermediate between the two parents involved in the fusion, as well as the tetraploid number of chromosomes in root tip preparations. These plants were shown to be somatic hybrids by isozyme and/or RAPD analysis.

All hybrids made with *S. disticha* seedlings were attacked by an unidentified fungus and died before they reached the height of 15 cm. This susceptibility may be caused by some negative interaction between the parental genomes in these hybrids, because the same result was observed in all of the hybrid plants, even though four *Citrus* cultivars and two *S. disticha* seedlings were used to make the hybrids. Figure 2

Table 14: Intergeneric somatic hybrids produced by protoplast fusion, with the respective number of regenerated plants.

Combination	Plants regenerated
'Succari' sweet orange + <i>S. disticha</i>	5
'Hamlin' sweet orange + <i>S. disticha</i>	5
'Valencia' sweet orange + <i>S. disticha</i>	3
'Nova' mandarin-tangelo + <i>S. disticha</i>	2
'Succari' sweet orange + <i>S. buxifolia</i>	35
'Succari' sweet orange + <i>A. ceylanica</i>	>150
'Succari' sweet orange + <i>F. limonia</i>	>60
'Nova' mandarin-tangelo + <i>C. gillettiana</i>	8

shows one regenerated somatic hybrid plant of each remaining parental combination under greenhouse conditions.

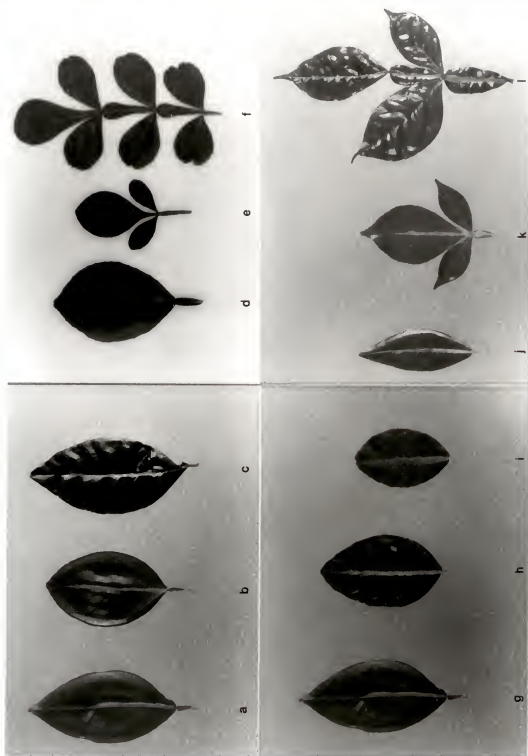
Representative leaf morphology of each of the remaining four wide hybrids and their parents is presented in Figure 3. Leaf morphology of the hybrids, as previously mentioned, is generally intermediate to that of the parental types.

RAPD analysis revealed complementary banding patterns in the somatic hybrids, indicating the presence of DNA from each parent in a corresponding hybrid (Figure 4). White dots in this figure show complementary, diagnostic bands from the parents that are present also in the somatic hybrid. Plants of the combinations 'Succari' + *S. buxifolia* and 'Nova' + *C. gillettiana* were regenerated from combined fusions experiments with Dr. Jude W. Grosser.



Figure 2: Intergeneric somatic hybrid plants regenerated from protoplast fusion: (a) 'Succari' sweet orange + *A. ceylanica*, (b) 'Succari' + *F. limonia*, (c) 'Succari' + *S. buxifolia*, (d) 'Nova' mandarin-tangelo + *C. gillettiana*.

Figure 3: Leaf morphology of intergeneric somatic hybrid plants and their respective parental types: (a) 'Succari' sweet orange, (b) 'Succari' + *A. ceylanica*, (c) *A. ceylanica*, (d) 'Succari', (e) 'Succari' + *F. limonia*, (f) *F. limonia*, (g) 'Succari', (h) 'Succari' + *S. buxifolia*, (i) *S. buxifolia*, (j) 'Nova' mandarin-tangelo, (k) 'Nova' + *C. gilletiana*, (l) *C. gilletiana*.



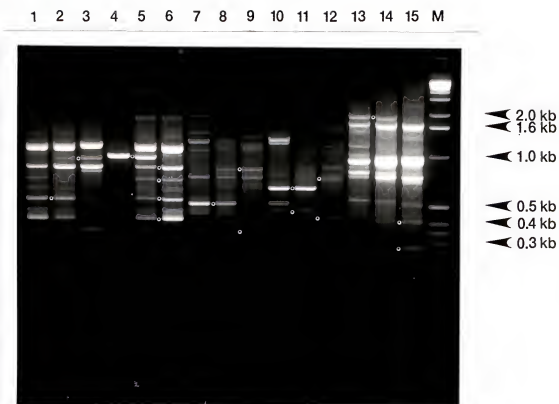


Figure 4: RAPD patterns of intergeneric somatic hybrid plants and their respective parental types. White dots show complementary, diagnostic bands from parents that are present in the somatic hybrid. Lanes 1 through 6 = W-15 primer, lanes 7 through 12 = A-7 primer, lanes 13 through 15, W-4 primer. Lanes 1, 6, 10, and 13 = 'Succari' sweet orange, lane 2 = 'Succari' + *S. buxifolia*, lane 3 = *S. buxifolia*, lane 4 = *F. limonia*, lane 5 = 'Succari' + *F. limonia*, lane 7 = 'Nova' mandarin-tangelo, lane 8 = 'Nova' + *C. gillettiana*, lane 9 = *C. gillettiana*, lanes 11 and 14 = 'Succari' + *A. ceylanica*, lanes 12 and 15 = *A. ceylanica*, M = DNA ladder.

The 'Succari' sweet orange + *S. buxifolia* somatic hybrid has shown adequate vigor. The first two hybrids of sweet orange with the genus *Severinia* previously produced (Grosser et al., 1988b, 1992b), have performed inconsistently in commercial rootstock trials, showing a lack of vigor and poor nutrition in some replications (Grosser, personal communication). Using a more vigorous selection of *S. buxifolia* that became available, the somatic hybrid reported herein was more vigorous than the previous two hybrids, and therefore has the potential to overcome their deficiencies. One possible explanation for the improved vigor and adaptation of this hybrid could be its much more fibrous root system, as compared to the previous two. It is hoped that this new hybrid will carry traits of horticultural interest for the industry, from the genus *Severinia*, such as cold resistance, salt and boron tolerance (Cooper, 1961; Cooper et al., 1957), *Phytophthora* resistance (Broadbent, 1969; Carpenter and Furr, 1962; Grimm and Hutchison, 1977; Hutchison and Grimm, 1973), and nematode resistance (Baines et al., 1960; Hutchison and O'Bannon, 1972).

The successful hybridization of 'Succari' sweet orange with *A. ceylanica* reported herein produced more than 150 plants, and they are more vigorous than the previous 'Hamlin' sweet orange + *Atalantia* hybrid (Louzada et al., 1993), from which only one hybrid plant of normal morphology was recovered. As mentioned previously, *Atalantia* is known to

perform well in wet soils, suggesting *Phytophthora* resistance, and it exhibits good cold-hardiness (Bitters et al., 1964; Campbell, 1979).

Citropsis offers strong resistance to *Phytophthora*-induced diseases (Swingle and Reece, 1967) and the burrowing nematode (Ford and Feder, 1960). The first two *Citrus* + *Citropsis* hybrids produced by Grosser and his colleagues (Grosser and Gmitter, 1990b; Grosser et al., 1990) exhibited some problems that are apparently not present in the 'Nova' mandarin tangelo + *C. gillettiana* hybrid reported herein. The hybrid of 'Hamlin' sweet orange + *C. gillettiana* has shown severe growth splits in the main trunk. These splits eventually heal over, but they reduce the growth and vigor of the trees, and make the trees more susceptible to herbicide damage (Grosser, personal communication). The Cleopatra mandarin + *C. gillettiana* hybrid has unexpectedly shown a high susceptibility to an undetermined leaf/stem fungal spotting disease that drastically reduces the vigor of the trees. It is not known if this problem is due to a negative genomic interaction, or to selection through somaclonal variation of a negative trait in the Cleopatra callus line used to make the hybrid (Grosser, personal communication). The new 'Nova' mandarin-tangelo + *C. gillettiana* hybrid is also much more vigorous than the previous two *Citrus* + *Citropsis* hybrids, and may avoid the problems associated with the earlier combination.

The hybrid between 'Succari' sweet orange + *F. limonia* produced during this research is the first report of successful somatic hybridization of *Citrus* with *Feronia* by any method. This is also the first report of successful somatic hybridization of *Citrus* with any member of the Wood-Apple group, Subtribe 3, Balsamocitrinae (hard-shelled citroid fruit trees), Tribe Citreae. As shown in Table 14, more than 60 hybrid plants were regenerated from this combination, and they have adequate vigor. *Feronia* is known to be drought tolerant, and due to its deciduous nature, it may be a source of genes for cold-hardiness (Swingle and Reece, 1967).

Interspecific Hybrids Produced and their Potential for Citrus Scion Improvement

Table 15 summarizes all the confirmed interspecific hybrid plants that were regenerated and currently growing in greenhouse conditions. Representative plants are shown in Figure 5. The combinations 'Succari' sweet orange + 'Murcott' tangor, 'Valencia' sweet orange + 'Murcott' tangor, 'Hamlin' sweet orange + 'Ponkan' mandarin, and 'Rohde Red Valencia' sweet orange + 'Dancy' tangerine were performed by Dr. Jude W. Grosser and provided to the project for analysis. As expected, all the somatic hybrid plants showed leaf morphology intermediate between the two parental types involved in the fusion (Figure 6), although this character was much more difficult to detect in close interspecific combinations.

Further, all hybrid plants were tetraploid ($2n=4x=36$), and of adequate vigor.

Leaf isozyme genotypes for *Per*, *Pgi*, and *Pgm-1* of the donor *Citrus* parents and the somatic hybrid plants are summarized in Table 16. Allelic designation was according to Moore and Castle (1988), for *Per*, and Torres et al. (1978), for *Pgi* and *Pgm-1*. Somatic hybridity of regenerants was verified by allelic complementation of 'Succari' with 'Dancy' tangerine (*Per*, *Pgm-1*), 'Page' tangelo (*Pgm-1*), and 'Ponkan' (*Per*, *Pgi*, and *Pgm-1*), and of 'Valencia' with 'Page' (*Pgm-1*). In the case of the combinations 'Succari' with 'Murcott', 'Hamlin' with 'Ponkan' and 'Valencia' with 'Murcott', allelic complementation was also observed in the form of allelic dosage effect (i.e. relatively greater intensity of the F band for FFFS plants vs. FS), especially for *Pgm-1*. Because of lack of polymorphism between 'Succari' sweet orange and 'Minneola' tangelo, further RAPD analysis was performed on this particular combination, which proved to be a somatic hybrid due to complementary banding patterns (Figure 7). The only plant from the 'Valencia' sweet orange + 'Minneola' tangelo combination was also proven to be a somatic hybrid, as revealed by RAPD analysis (Figure 8). For these combinations, the use of at least two primers was necessary to show complementation. P-10 primer shows a banding pattern indicating the presence of sweet orange DNA in the respective somatic hybrids, whereas X-18 primer shows the presence of

Table 15: Interspecific somatic hybrids produced by protoplast fusion, with the respective number of regenerated plants.

Combination	Plants regenerated
'Succari' sweet orange + 'Dancy' tangerine	39
'Succari' sweet orange + 'Minneola' tangelo	53
'Succari' sweet orange + 'Murcott' tangor	2
'Succari' sweet orange + 'Page' tangelo	20
'Succari' sweet orange + 'Ponkan' mandarin	31
'Hamlin' sweet orange + 'Ponkan' mandarin	17
'Valencia' sweet orange + 'Minneola' tangelo	1
'Valencia' sweet orange + 'Murcott' tangor	15
'Valencia' sweet orange + 'Page' tangelo	11
'Rohde Red Valencia' swt. orange + 'Dancy' tangerine	2

Table 16: Leaf isozyme genotypes of the donor *Citrus* parents and the somatic hybrid plants for peroxidase (Per), phosphoglucose isomerase (Pgi), and phosphoglucomutase (Pgm-1).

	Per	Pgi	Pgm-1
<u>Donor Citrus parents:</u>			
'Succari' sweet orange (Su)	FF	MS	FS
'Hamlin' sweet orange (Ha)	FF	MS	FS
'Valencia' sweet orange (Va)	FF	MS	FS
'Dancy' tangerine (Da)	FS	MM	FF
'Minneola' tangelo (Mi)	FF	MS	FS
'Murcott' tangor	FF	MM	FF
'Page' tangelo (Pg)	FF	MS	IS
'Ponkan' mandarin (Pk)	SS	MM	FF
<u>Somatic hybrid combinations:</u>			
(Su) + (Da)	FFFS	MMMS	FFFS
(Su) + (Mi)	FFFF	MMSS	FFSS
(Su) + (Mu)	FFFF	MMMS	FFFS
(Su) + (Pg)	FFFF	MMSS	FISS
(Su) + (Pk)	FFSS	MMMS	FFFS
(Ha) + (Pk)	FFSS	MMMS	FFFS
(Va) + (Mu)	FFFF	MMMS	FFFS
(Va) + (Pg)	FFFF	MMSS	FISS

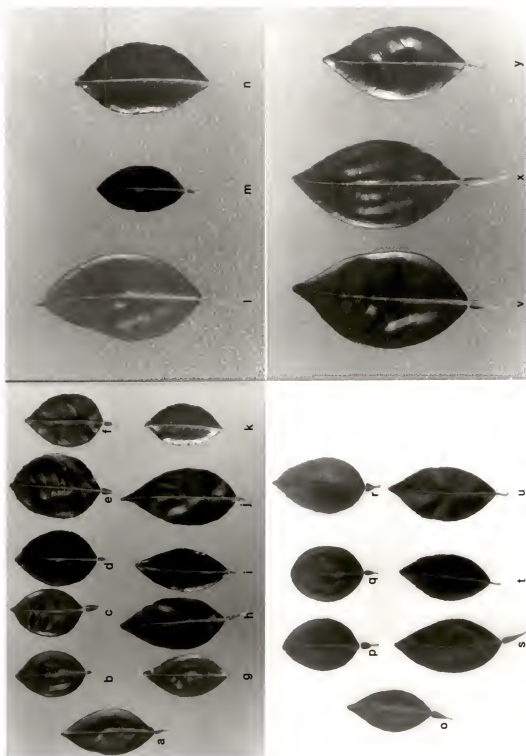


Figure 5: Interspecific somatic hybrid plants regenerated from protoplast fusion: (a) 'Succari' sweet orange + 'Dancy' tangerine, (b) 'Succari' + 'Minneola' tangelo, (c) 'Succari' + 'Murcott' tangor, (d) 'Succari' + 'Page' tangelo, (e) 'Succari' + 'Ponkan' mandarin, (f) 'Hamlin' sweet orange + 'Ponkan' mandarin.



Figure 5--continued: Interspecific somatic hybrid plants regenerated from protoplast fusion: (g) 'Valencia' sweet orange + 'Minneola' tangelo, (h) 'Valencia' + 'Murcott' tangor, (i) 'Valencia' + 'Page' tangelo, (j) 'Rohde Red Valencia' sweet orange + 'Dancy' tangerine.

Figure 6: Leaf morphology of interspecific somatic hybrid plants and their respective parental types. (a) 'Succari' sweet orange, (b) 'Succari' + 'Dancy' tangerine, (c) 'Succari' + 'Minneola' tangelo, (d) 'Succari' + 'Murcott' tangor, (e) 'Succari' + 'Page' tangelo, (f) 'Succari' + 'Ponkan' mandarin, (g) and (y) 'Dancy', (h) and (s) 'Minneola', (i) and (t) 'Murcott', (j) and (u) 'Page', (k) and (n) 'Ponkan', (l) 'Hamlin' sweet orange, (m) 'Hamlin' + 'Ponkan' mandarin, (o) 'Valencia' sweet orange, (p) 'Valencia' + 'Minneola', (q) 'Valencia' + 'Murcott', (r) 'Valencia' + 'Page', (v) 'Rohde Red Valencia' sweet orange, (x) 'Rohde Red Valencia' + 'Dancy'.



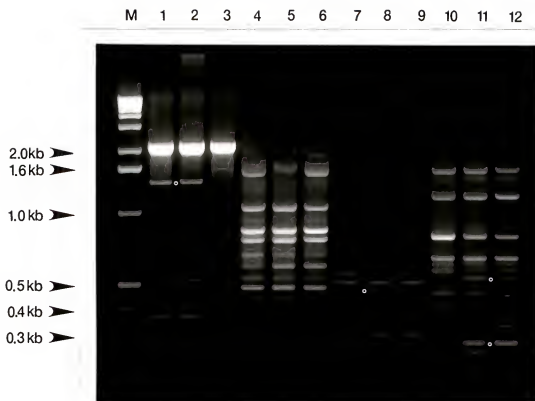


Figure 7: RAPD patterns of interspecific somatic hybrid 'Succari' sweet orange plus 'Minneola' tangelo and its respective parental types. White dots show complementary, diagnostic bands from parents that are present in the somatic hybrid. Lanes 1 through 3 = P-10 primer, lanes 4 through 6 = U-17 primer, lanes 7 through 9 = W-5 primer, lanes 10 through 12 = X-18 primer. Lanes 1, 4, 7, and 10 = 'Succari', lanes 2, 5, 8, and 11 = 'Succari' + 'Minneola', lanes 3, 6, 9, and 12 = 'Minneola', M = DNA ladder.

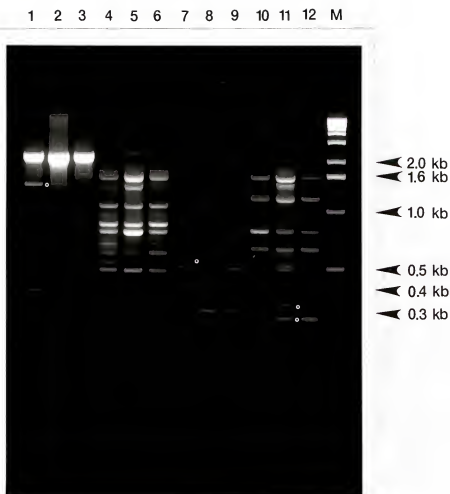


Figure 8: RAPD patterns of interspecific somatic hybrid 'Valencia' sweet orange plus 'Minneola' tangelo and its respective parental types. White dots show complementary, diagnostic bands from parents that are present in the somatic hybrid. Lanes 1 through 3 = P-10 primer, lanes 4 through 6 = U-17 primer, lanes 7 through 9 = W-5 primer, lanes 10 through 12 = X-18 primer. Lanes 1, 4, 7, and 10 = 'Valencia', lanes 2, 5, 8, and 11 = 'Valencia' + 'Minneola', lanes 3, 6, 9, and 12 = 'Minneola', M = DNA ladder.

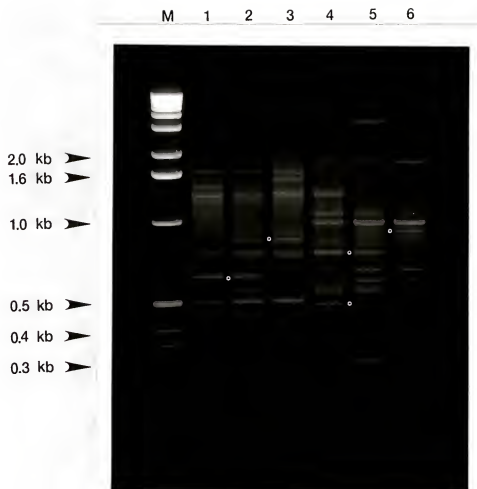


Figure 9: RAPD patterns of interspecific somatic hybrid 'Rohde Red Valencia' sweet orange plus 'Dancy' tangerine and its respective parental types. White dots show complementary, diagnostic bands from parents that are present in the somatic hybrid. Lanes 1 through 3 = A-7 primer, lanes 4 through 6 = A-8 primer. Lanes 1 and 4 = 'Rohde Red Valencia', lanes 2 and 5 = 'Rohde Red Valencia' + 'Dancy', lanes 3 and 6 = 'Dancy', M = DNA ladder.

'Minneola' tangelo DNA (Figures 7 and 8). The complementation of bands is also shown with other primers for the combination 'Rohde Red Valencia' sweet orange + 'Dancy' mandarin (Figure 9). White dots in Figures 7, 8, and 9 show diagnostic bands, present in the DNA of donor parents and in the DNA of the somatic hybrid. Figure 7 also shows no polymorphism for the primer U-17 between the two parents, but this polymorphism does not appear in the DNA of the resultant somatic hybrid. Also, very faint bands for the primer W-5 are not desirable and make the diagnosis more difficult.

Mandarin hybrids, e.g., tangors [hybrids of sweet orange with mandarin] and tangelos [grapefruit X mandarin hybrids] are among the most important *Citrus* cultivars for fresh fruit, but many of these cultivars have an undesirable seed content (Saunt, 1990). Triploidy may be exploited to produce seedless citrus fruit. Some examples include 'Tahiti' (or 'Persian') lime (*C. aurantifolia* [Christ.] Swing.), and 'Melgold' and 'Oroblanco' pummelo X grapefruit hybrids (Krug, 1943; Krug and Bacchi, 1943; Soost and Cameron, 1980, 1985).

Several interspecific somatic hybrids have been produced in attempts to combine cultivar characteristics and overcome barriers to sexual hybridization, such as sterility, polyembryony, and low seed production from cultivars such as 'Washington' navel orange (*C. sinensis*) and 'Hayashi' satsuma mandarin (*C. unshiu*) (Kobayashi et al., 1988b). Other *Citrus* somatic hybrids produced with this objective include

'Washington' navel orange + 'Murcott' tangor (Kobayashi and Ohgawara, 1988), 'Trovita' sweet orange + 'Hayashi' satsuma mandarin, 'Bahia' navel orange + 'Marsh' grapefruit (*C. paradisi*) (Ohgawara et al., 1989), 'Thompson' grapefruit + 'Murcott' tangor (Grosser et al., 1992b), 'Nova' tangelo ['Clementine' mandarin (*C. reticulata*) X 'Orlando' tangelo (*C. paradisi* X *C. reticulata*)] + 'Succari' sweet orange, and 'Hamlin' sweet orange + 'Dancy' tangerine (Grosser et al., 1992a). Although somatic hybrids of scion cultivars may have potential for direct use as a scion, the most important role of somatic hybridization in *Citrus* scion improvement likely will be the production of interspecific allotetraploid somatic hybrids ($2n=4x=36$) that combine the genomes of complementary parents (Gmitter et al., 1992; Grosser, 1993). Such tetraploid hybrids can be hybridized with monoembryonic diploid female parents ($2n=2x=18$) to generate triploid zygotic progeny that should produce seedless fruit. Although seeds that contain triploid zygotic embryos from crosses of diploid females with tetraploid pollen parents generally are not well developed and their germination rate is very low, embryo rescue and in vitro culture can overcome this difficulty (Starrantino and Recupero, 1981).

High levels of acid in the fruit of progeny from crosses involving mandarins has been a problem in *Citrus* breeding programs, according to Soost and Cameron (1969). In a later report, Soost and Cameron (1975), mention that unacceptably

high levels of acid in hybrid citrus fruit frequently result from crosses of parents with medium acid levels. Grosser et al., (1992a) have described the good horticultural characteristics of the 'Succari' sweet orange and its potential when utilized as a parent in somatic hybridization. 'Succari' sweet orange is a vigorous, early-ripening cultivar with good fruit color. It is virtually acidless at maturity with sugar:acid ratio of 90-100:1. According to Barrett, (1990), 'Succari' sweet orange transmits low acidity to its progeny, more so than other low acid oranges. Therefore, the use of tetraploid breeding parents containing 'Succari' sweet orange parentage in interploid crosses may increase the percentage of triploid zygotic progeny that produce fruit with acceptable acid levels. Additional reasons for the choice of 'Succari' as one of the parents in somatic hybridization experiments have been previously discussed.

All hybrids reported herein have been grafted to *Citrus* rootstocks to hasten flowering. They have been planted in the field to expedite their use as pollen parents in sexual hybridization with selected diploid monoembryonic seed parents.

Utilization and Testing of Random Amplified Polymorphic DNA (RAPD) Analysis as a Method for Intergeneric and Interspecific Citrus Somatic Hybrid Identification and Verification

The utilization and testing of RAPD analysis, recently developed by Xiao et al. (personal communication) for *Citrus*

somatic hybrid identification and verification, was one of the objectives of this research. For most of the hybrids tested, a preliminary screening of primers was necessary to identify several polymorphisms between the two parents involved in the fusion. Once the most appropriate primer or primers were identified, a second PCR reaction and gel were prepared including a sample of the hybrid DNA. As expected, the screening of primers for intergeneric combinations was easier than for interspecific combinations, because of the greater genetic distance between the two parents. This is the first report of the use of RAPD analysis for intergeneric somatic hybrid identification. The use of RAPD analysis for intergeneric somatic hybrid identification overcomes not only the main disadvantage of isozyme analysis, i.e., minimal levels of polymorphism, but also another inconvenience of this technique, which is the lack of resolution often encountered with starch gel electrophoresis, especially in cases involving *Citrus* relatives. These species have higher concentrations of phenols and other substances in the leaves that can interfere with the movement of the proteins during electrophoresis, and in subsequent enzyme reactions (Gmitter, personal communication).

RAPD analysis has been shown to be useful for identification and verification of interspecific somatic hybrids. Xiao et al. (personal communication) recently have developed and adapted this technique for *Citrus*, and the

further testing of this protocol, for the hybrids produced in this research, confirms its reliability and advantages. This is the case of some of the somatic hybrids produced in this present research, such as the interspecific combinations 'Succari' sweet orange + 'Minneola' tangelo and 'Valencia' sweet orange + 'Minneola' tangelo. Preliminary isozyme analysis for these combinations revealed no polymorphism between the two respective parents involved in the fusion for three isozyme systems (*Per*, *Pgi*, and *Pgm-1*).

RAPD analysis, like RFLP analysis, is a direct and accurate method, in which differences are revealed at the DNA level, providing the ultimate genetic evidence for hybrid verification. In contrast, morphological traits can be influenced more or less by the environment, leading, in some cases, to false conclusions. Because the amount of DNA necessary for RAPD analysis is much lower than RFLP analysis, small plantlets, embryos, somatic hybrid calli, or even single cells (Brown et al., 1993) can be analyzed.

Xiao et al. (personal communication) indicated several limitations to the use of RAPD analysis for hybrid identification and verification. RAPD markers, as opposed to isozymes or RFLPs, are dominant, so they are somewhat less informative because homozygotes and heterozygotes possessing a given marker are indistinguishable. Another limitation of RAPD analysis is that the origins of most RAPD fragments have not been determined by following the segregation of these

fragments in hybrid families (Gmitter, personal communication). Therefore, the use of RAPDs to verify somatic hybridity poses some risk, because a given fragment may be of cytoplasmic rather than nuclear origin, and it is possible that a cybrid may be identified incorrectly as a somatic nuclear hybrid. In some cases, there is polymorphism between the two parents involved in the fusion, but this polymorphism does not appear in the somatic hybrid. This case can be exemplified with the somatic hybrid 'Succari' sweet orange + 'Minneola' tangelo, with the primer U-17. As shown in Figure 7 (lanes 4 through 6), DNA from sweet orange produced a unique band (approximately 0.7 kb), which 'Minneola' does not have. The somatic hybrid also should have this band, but it does not. There are other cases in which there is sufficient polymorphism to prove hybridity, but some bands present in the DNA from the donor parents are also missing in the DNA from the somatic hybrid. This is the case illustrated in Figure 4 (lanes 10 through 12) for the somatic hybrid 'Succari' sweet orange + *Atalantia ceylanica*. DNA amplification from sweet orange with the A-7 primer shows three bands (two of them approximately 1.3 kb and another one approximately 0.5 kb) that are not present in the somatic hybrid DNA. Nevertheless, there are some complementary polymorphisms that confirm hybridity. These bands may have been lost through DNA recombination (nuclear or cytoplasmic DNA) or they may have been DNA sequences that were lost in the fusion.

There are other examples where the somatic hybrid DNA amplification shows extra bands that are not present in either of the parents. This is the case of the somatic hybrid 'Valencia' sweet orange + 'Minneola' tangelo DNA amplified with the primer U-17 (Figure 8, lanes 4 through 6), which shows an extra band (approximately 1.5 kb) not present in the DNA amplified from either donor parent. Again, a possible explanation for this unexpected result could be DNA recombination (nuclear or cytoplasmic DNA). The origin of these extra bands has not been determined. In spite of these occasional unexpected results, RAPD analysis has been shown to be a routine, reliable and simple procedure for the identification and verification of intergeneric and interspecific *Citrus* somatic hybrids.

SUMMARY AND CONCLUSIONS

I have reported herein the production of four new intergeneric somatic hybrids of *Citrus* and its relatives with direct potential for rootstock improvement. The combinations are 'Succari' sweet orange with *S. buxifolia*, *A. ceylanica*, and *F. limonia*; and 'Nova' mandarin-tangelo + *C. gillettiana*. All the combinations involve a different genus with some traits of interest to be incorporated into the *Citrus* gene pool. The hybrid 'Succari' sweet orange + *F. limonia* is the first report of hybridization with *Feronia* ever, by any method. All the intergeneric hybrids produced in this research, with the exception of the *Citrus* + *S. disticha* hybrids which did not survive, have been propagated, and most of them are planted in commercial trials to evaluate their rootstock potential. Grafted seed-trees of each hybrid are also being grown out to determine if they can be propagated by seed. Information from field trials will provide valuable feedback regarding the selection of parents for future somatic hybridization experiments.

I have also reported the production of ten new interspecific *Citrus* somatic hybrids for scion development as follows: 'Succari' sweet orange with 'Dancy' tangerine, 'Minneola' tangelo, 'Murcott' tangor, 'Page' tangelo, and

'Ponkan' mandarin; 'Valencia' sweet orange with 'Minneola' tangelo, 'Murcott' tangor, and 'Page' tangelo; 'Hamlin' sweet orange with 'Ponkan' mandarin; and 'Rohde Red Valencia' sweet orange with 'Dancy' tangerine. All of these hybrids have been grafted to *Citrus* rootstocks to hasten flowering, and they have been planted in the field to expedite their use as pollen parents in sexual hybridization with selected diploid monoembryonic seed parents. Improved seedless mandarin cultivars will allow citrus growers to expand their national marketing potential, and to become competitive in the international markets that do not accept seedy fruit, regardless of quality.

It is important to emphasize the difficult task in accomplishing somatic hybridization, in spite of the fact that *Citrus* has become a model for somatic hybridization of woody plants. In particular, when dealing with intergeneric combinations, optimization of all steps of the hybridization must be accomplished, including: the selection of good potential sources of protoplasts of the *Citrus* relatives; the adaptation of the protocols for isolation, purification, fusion, and protoplast culture; and conditions for embryo induction and plant regeneration. Somatic hybridization has proven to be a technique in which very many experiments must be performed and repeated to achieve the desired goals. This can be shown best by the fact that over 120 separate fusion

attempts were required to produce somatic hybrids from fourteen distinct parental combinations.

Additional important accomplishments in this research are the adaptation and successful development of alternative sources of protoplasts from the *Citrus* relatives for use in the somatic hybridization experiments, and the utilization and testing of RAPD analysis, recently developed and adapted for *Citrus* somatic hybrid identification and verification. Although the exhaustive work in searching for non-embryogenic calli from the *Citrus* relatives did not result in many successes, the adaptation of the protocol for protoplast isolation from greenhouse-grown leaves, and the protoplast isolation from in vitro seedling leaves, were important advances. RAPD analysis proved to be a powerful and reliable tool for somatic hybrid identification and verification.

APPENDIX

TISSUE CULTURE AND PROTOPLAST MEDIA

MT Basal Medium Stock Solutions (Murashige and Tucker, 1969)

<u>MT Macronutrient Stock (50 X)</u>	<u>g/liter</u>
NH_4NO_3	82.5
KNO_3	95.0
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	18.5
KH_2PO_4 (monobasic)	7.5
K_2HPO_4 (dibasic)	1.0

<u>MT Micronutrient Stock (100X)</u>	<u>g/liter</u>
H_3BO_3	0.62
$\text{MnSO}_4 \cdot \text{H}_2\text{O}$	1.68
$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	0.86
KI	0.083
$\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$	0.025
$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	0.0025
$\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$	0.0025

<u>MT Iron Stock (200 X)</u>	<u>g/liter</u>
Na_2EDTA	7.45
$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	5.57

<u>MT Vitamin Stock (100 X)</u>	<u>g/liter</u>
myo-inositol	10.0
thiamine-HCl	1.0
pyridoxine-HCl	1.0
nicotinic acid	0.5
glycine	0.2
<u>MT Calcium Stock (66 X)</u>	<u>g/liter</u>
$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	29.33

BH3 Stock Solutions

<u>BH3 Macronutrient Stock (100 X)</u>	<u>g/liter</u>
MgSO_4	37
KH_2PO_4 (monobasic)	15
K_2HPO_4 (dibasic)	2
KCl	150
<u>BH3 Multivitamin A Stock (100 X)</u>	<u>g/100 ml</u>
Calcium pantothenate	0.05
Ascorbic acid	0.1
Choline chloride	0.05
p-Aminobenzoic acid	0.001
Folic acid	0.02
Riboflavin	0.01
Biotin	0.001
<u>BH3 Multivitamin B Stock (100 X)</u>	<u>g/100 ml</u>
Retinol (Vitamin A)*	0.001
Cholecalciferol (Vitamin D ₃)*	0.001
Vitamin B ₁₂	0.002
* insoluble in water, soluble in ETOH	
<u>BH3 KI Stock (100 X)</u>	<u>g/100 ml</u>
KI	0.075

<u>BH3 Organic Acid Stock (50 X)</u>	<u>g/100 ml</u>
Sodium pyruvate	0.1
Citric acid	0.2
Malic acid	0.2
Fumaric acid	0.2
<u>BH3 Sugar + Sugar Alcohol Stock (100 X)</u>	<u>g/100 ml</u>
Fructose	2.5
Ribose	2.5
Xylose	2.5
Mannose	2.5
Rhamose	2.5
Cellobiose	2.5
Galactose	2.5
Mannitol	2.5

Media composition:

EME (Regular EME, 0.146M)

MT Macro Stock (50 X)	20 ml/l
MT Micro Stock (100 X)	10 ml/l
MT Vitamin Stock (100 X)	10 ml/l
MT Calcium Stock (66 X)	15 ml/l
MT Iron Stock (200 X)	5 ml/l
Sucrose*	50 g/l
Malt Extract	0.5 g/l
Agar**	8 g/l

Adjust pH to 5.8 with KOH, before adding agar
 Filter sterilize (0.2 μ unit) if used for protoplast culture;
 for all other uses autoclave 15 minutes at 15 psi.

- * for 0.6 M EME use 205.38 g/l sucrose
- for 0.7 M EME use 239.61 g/l sucrose
- ** use high grade sucrose for protoplast culture
- omit agar for liquid EME

1/2-1/2 EME

MT Macro Stock (50 X)	10 ml/l
BH3 Macro Stock (100 X)	5 ml/l
MT Micro Stock (100 X)	10 ml/l
MT Vitamin Stock (100 X)	10 ml/l
MT Calcium Stock (66 X)	15 ml/l
MT Iron Stock (200 X)	5 ml/l
Sucrose	50 g/l
Malt Extract	0.5 g/l
Glutamine	1.55 g/l
Agar*	8 g/l

Adjust pH to 5.8 with KOH, before adding agar

Autoclave 15 minutes at 15 psi.

* omit agar for liquid EME

1500

MT Macro Stock (50 X)	20 ml/l
MT Micro Stock (100 X)	10 ml/l
MT Vitamin Stock (100 X)	10 ml/l
MT Calcium Stock (66 X)	15 ml/l
MT Iron Stock (200 X)	5 ml/l
Sucrose	50 g/l
Malt Extract	1.5 g/l
Agar	8 g/l

Adjust pH to 5.8 with KOH, before adding agar

B+

MT Macro Stock (50 X)	20 ml/l
MT Micro Stock (100 X)	10 ml/l
MT Vitamin Stock (100 X)	10 ml/l
MT Calcium Stock (66 X)	15 ml/l
MT Iron Stock (200 X)	5 ml/l
Coconut water	20 ml/l
Sucrose	25 g/l
Coumarin Stock (1.46 mg/ml)	10 ml
NAA Stock (1 mg/ml)	20 μ l
Agar	8 g/l

Adjust pH to 5.8 with KOH, before adding agar

Autoclave 15 minutes at 15 psi.

After autoclaving add filter sterilized:

GA ₃ Stock (10 mg/ml)	10 ml/l
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DBA3

MT Macro Stock (50 X)	18 ml/l
MT Micro Stock (100 X)	9 ml/l
MT Vitamin Stock (100 X)	9 ml/l
MT Calcium Stock (66 X)	15 ml/l
MT Iron Stock (200 X)	5 ml/l
Coconut water	20 ml/l
Sucrose	25 g/l
Malt extract	1.5 g/l
2,4-D Stock (66.3 mg/l)	152 μ l
6-BAP Stock (1 mg/ml)	3 ml
Agar	8 g/l

Adjust pH to 5.8 with KOH, before adding agar

Autoclave 15 minutes at 15 psi.

CW28

MT Macro Stock (50 X)	20 ml/l
MT Micro Stock (100 X)	10 ml/l
MT Vitamin Stock (100 X)	10 ml/l
MT Calcium Stock (66 X)	15 ml/l
MT Iron Stock (200 X)	5 ml/l
Sucrose	25 g/l
Malt extract	0.5 g/l
6-BAP Stock (1 mg/ml)	500 µl
IBA Stock (1 mg/ml)	250 µl
Adenine	0.030 g
Agar	8 g/l

Adjust pH to 5.8 with KOH, before adding agar

Autoclave 15 minutes at 15 psi.

After autoclaving add filter sterilized:

GA ₃ Stock (10 mg/ml)	10 ml/l
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CW32

MT Macro Stock (50 X)	20 ml/l
MT Micro Stock (100 X)	10 ml/l
MT Vitamin Stock (100 X)	10 ml/l
MT Calcium Stock (66 X)	15 ml/l
MT Iron Stock (200 X)	5 ml/l
Sucrose	25 g/l
Malt extract	0.5 g/l
6-BAP Stock (1 mg/ml)	500 µl
IBA Stock (1 mg/ml)	250 µl
Adenine	0.030 g
Coumarin Stock (1.46 mg/ml)	6.8 ml/l
Agar	8 g/l

Adjust pH to 5.8 with KOH, before adding agar

Autoclave 15 minutes at 15 psi.

After autoclaving add filter sterilized:

GA ₃ Stock (10 mg/ml)	10 ml/l
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RMAN (Rooting medium)

MT Macro Stock (50 X)	10 ml/l
MT Micro Stock (100 X)	5 ml/l
MT Vitamin Stock (100 X)	5 ml/l
MT Calcium Stock (66 X)	15 ml/l
MT Iron Stock (200 X)	5 ml/l
NAA Stock (279.3 mg/l)	72 μ l
Activated charcoal	0.5 g
Sucrose	25 g/l
Agar	8 g/l

Adjust pH to 5.8 with KOH
Autoclave 15 minutes at 15 psi.

BH3 (0.6 M)

BH3 Macro Stock (100 X)	10 ml/l
MT Micro Stock (100 X)	10 ml/l
MT Vitamin Stock (100 X)	10 ml/l
MT Calcium Stock (66 X)	15 ml/l
MT Iron Stock (200 X)	5 ml/l
BH3 Multivitamin A Stock (100 X)	2 ml/l
BH3 Multivitamin B Stock (100 X)	1 ml/l
BH3 KI Stock (100 X)	1 ml/l
BH3 Sugar Alcohol Stock (100 X)	10 ml/l
BH3 Organic Acid Stock (50 X)	20 ml/l
Coconut water	20 ml/l
Malt Extract	1 g/l
Sucrose ^(*)	51.35 g/l
Mannitol	81.99 g/l
Glutamine	3.1 g/l
Casein Enzyme Hydrolysate	0.25 g/l

Adjust pH to 5.7 with KOH
Filter sterilize with 0.2 μ unit
Store in the dark

(*) For 0.7 M BH3 increase the sucrose to 85.56 g/l

CPW Salts (Freason et al., 1973)Stock 1KH₂PO₄KNO₃MgSO₄

KI

CuSO₄Stock conc.

0.2720 g/100 ml

1.0000 g/100 ml

2.5000 g/100 ml

0.0016 g/100 ml

0.000025 g/100 ml

Stock 2CaCl₂Stock conc.

1.5 g/100 ml

Add 1 ml.100 ml of each stock

Adjust pH to 5.8 with KOH

Filter sterilize with 0.2 μ unit

Store in cold

For CPW13MAN add 13 g/100 ml of mannitol

For CPW25SUC add 25 g/100 ml of sucrose

Store in cold

Enzyme Solution

Mannitol (0.7 M)

CaCl₂ (24.5 mM)NaH₂PO₄ (0.92 mM)

M.E.S. (6.15 mM)

1% Cellulase (Onuzuka R.S.)

1% Macerase

0.2% Pectolyase Y-23

5.12 g/40 ml

0.144 g/40 ml

0.0044 g/40 ml

0.048 g/40 ml

0.4 g/40 ml

0.4 g/40 ml

0.08 g/40 ml

Adjust pH to 5.6 with KOH

Filter sterilize with 0.2 μ unit

PEG Solution

Polyethylene Glycol (1500 MW) ^(*)	40 g/100 ml
CaCl ₂ * 2H ₂ O	0.97 g/100 ml
Glucose	5.41 g/100 ml

(*) Deionize with AG501-x8 resin
 Adjust pH to 6.0 with KOH
 Filter sterilize with 0.2 μ unit

Solution A

Glucose	7.2 g/100 ml
CaCl ₂ * 2H ₂ O	0.97 g/100 ml
DMSO	10 ml/100 ml

Adjust pH to 6.0 with KOH

Solution B

Glycine	2.25 g/100 ml
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Adjust pH to 10.5 with KOH pellets

Filter sterilize all solutions with 0.2 μ units and store in the dark

Protoplasts Media for Osmoticum Reduction1-1-1

- 1 part 0.6 M BH3
- 1 part 0.6 M EME
- 1 part regular EME (0.146 M)

pH = 5.7

1-2

- 1 part 0.6 M BH3
- 2 parts regular EME (0.146 M)

pH = 5.7

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BIOGRAPHICAL SKETCH

Francisco de Assis Alves Mourão Filho was born November 12, 1964, in São Paulo, SP, Brazil. He lived in São Paulo, where he attended first grade through high school, graduating from Colégio Rio Branco in 1981. He then attended the University of São Paulo, at Piracicaba, where he received his Bachelor of Science degree in Agricultural Engineering in 1985.

Francisco then moved to Botucatu, SP, in 1986, where he pursued the Master of Science in Agronomy-Horticulture degree, at São Paulo State University, concluding his program in 1990. Throughout his Master's program he worked as a Citrus consultant for a Citrus Corporative in Araraquara, SP, and afterward as an assistant professor of horticulture at São Paulo State University in Ilha Solteira, SP.

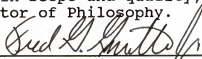
Francisco was hired in 1990 by University of São Paulo at Piracicaba, SP, as an assistant professor in the Department of Horticulture for teaching, research and extension in fruit crops and horticulture. He obtained his leave to attend the University of Florida in 1992. After concluding his program at this university, Francisco is going back to his country and his job at the University of São Paulo.

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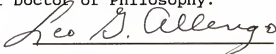
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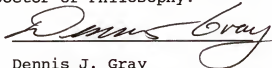
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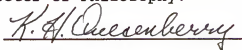
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